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The Journal of Biochemistry, Vol. 40, No. 1.

DETERMINATION OF ACTIVE HYDROGENS OF GUANOSINE AND YEAST RIBONUCLEIC ACID WITH DEUTERIUM OXIDE

By MASAO UCHIDA AND KATASHI MAKINO

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(Received for publication, September 8, 1952)

Microdetermination of active hydrogen with deuterium oxide was proposed in 1936 by R.J. Williams (*1*) and first applied to hydroxyproline and urea. It consists merely in dissolving the substances to be analysed in deuterium oxide, evaporating to dryness and determining the increase in weight due to the replacement of active hydrogens by deuterium. Bonhoeffer and Brown, (*2*) Klar, (*3*) and Williams (*1*) showed that the hydrogens of -OH, $-NH_2$ and -NH radicals in organic compounds were exchanged by simply dissolving them in deuterium oxide, while hydrogen of $-CH_3$, $-C_2H_5$ and $-C_6H_5$ was not exchanged at all. Since the principle is so simple and the manipulation involves nothing beyond drying and weighing, and the action is so mild, the authors have applied this method to the nucleic acid chemistry. After the applicability of this method was first checked on a nucleoside, guanosine, the authors used it to determine active hydrogens of yeast ribonucleic acid. The experiment showed that the number of active hydrogen of guanosine was 6, corresponding to the theoretical number, while the sodium nucleate was found to have 11–12 active hydrogens for every four phosphate groups.

EXPERIMENTAL

Experiment with Guanosine: First of all, two moles of crystal water of pure guanosine were previously expelled by heating at 110° in a pressure of 5 mm.Hg. Then 55.592 and 33.635 mg. of guanosine in small weighing bottles were dissolved in 1.0 ml. of 99.5 per cent deuterium oxide by heating, and then carefully evaporated and dried to constant weight in a pressure of 5 mm.Hg. at 110°; it was weighed again. (Table I).

The guanosine which underwent the above treatment was dissolved again in 1.0 ml. of 99.5 per cent deuterium oxide, evaporated and dried to constant weight and weighed again as above mentioned. But no increase was found. Therefore, the ex-

TABLE I

Number of Active Hydrogen of Guanosine

Guanosine	Increase after dissolving in deuterium oxide	Theoretical increase for one hydrogen	Number of active hydrogen
mg. 55.592,2	*mg.* 1.170	*mg.* 0.198,0	5.90 (≑6)
33.635,0	0.708	0.119,5	5.92 (≑6)

change of active hydrogens of guanosine seemed to accomplish by dissolving it in 99.5 per cent deuterium oxide only once.

Experiment with Yeast Nucleic Acid: Yeast ribonucleic acid employed was prepared from yeast by the method of Baumann and purified according to Makino (*4*) and has the following analytical data: N, 14.47%, P, 8.60%, therefore P:N=1:1.682. The acid was neutralized with *N*/10-NaOH using phenolphthaleine as indicator and evaporated and dried to constant weight at 110° in a pressure of 5 mm.Hg. 96.580 and 99.001 mg. of this sample were weighed out. Each sample was dissolved into 0.5 ml. of deuterium oxide and was carefully evaporated and dried to constant weight in a pressure of 5 mm.Hg. at 110° for about 18 hours. Weighing was repeated and the increase in weight was determined (Table II).

TABLE II

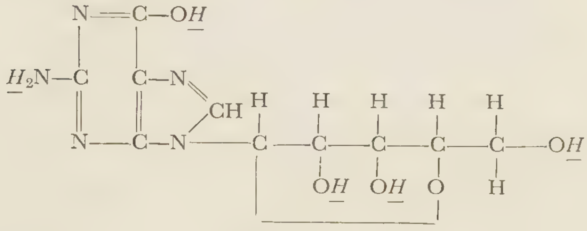
Number of Active Hydrogen of Yeast Ribonucleic Acid

Sodium salt of yeast nucleic acid	Increase after dissolving in deutrium oxide	Theoretical increase for one hydrogen	Number of active hydrogen per four phosphate groups
mg. 96.580	*mg.* 0.802,0	*mg.* 0.070,8	11.33
99.001	0.869,0	0.072,6	11.98
		Average	11.65

The sample which underwent the above treatment was dissolved again in 0.5 ml. of 99.5 per cent deuterium oxide. It was then evaporated and dried to constant weight and weighed again after treating as above. However no increase in weight was found. It seems that the exchange of active hydrogens of the sodium salt was completed by dissolving it in deuterium oxide only once.

DISCUSSION

The structure of guanosine is to be shown by the following Formula 1.

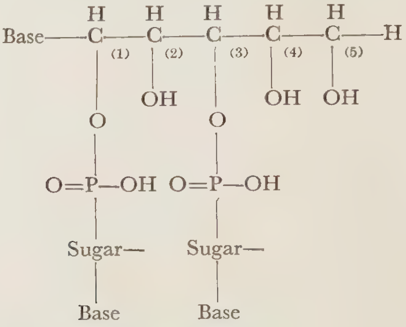


FORMULA 1

Theoretically number of its active hydrogens is 6 which are underlined in the above formula, and our experimental data coincide with it as shown in the above table (Table I).

In certain yeast ribonucleic acid (especially, of Merck) the ratio of four bases (adenine, guanine, cytosine and uracil) contained in them is equimolecular (Bacher and Allen, (*5*) Makino and Uchida (*6*). Levene and Simms (*7*) showed that the amino- and hydroxyl-groups of adenine, cytosine, guanine and uracil exist in free state. Levene and Jacobs (*8*), and Falconer, Gulland, Hobday and Jackson (*9*) indicated that their amino-groups can be determined by Van Slyke's method. Bredereck, Koethning and Lehmann (*10*) prepared a desaminated nucleic acid without decomposing its internucleotides union. So it seems that the amino- and hydroxyl-groups have nothing to do with the internucleotidal esterlinkage. On the other hand our samples of yeast ribonucleic acids (especially, that of Merck) have a molecular weight corresponding to a tetranucleotide (*11*) and show to be four basic and when it was decomposed into four nucleotides by alkali an increase of 4 acidic groups are afresh found (*12*). According to the cyclic formula the active hydrogens of its tetrasodium salt are 12 while our experimental results indicated that the number is 11–12 as shown in the above table (Table II).

As the addendum to the above experiment we intended to study the attitude of yeast nucleic acid toward periodate oxidation in order to examine whether the possiblity exists or not that a inter-mononucleotide ester linkage takes place between a hydroxylgroup of position (1) of one of mononucleotides and a phosphoric acid group of the other monounucleotide and accordingly hydroxyl-groups of position (4) and (5) of its sugar are free as Formula 2.



FORMULA 2

But the experimental results indicated that no consumption of oxygen was found when periodate was added to the solution of sodium salt of yeast ribonucleic acid.

SUMMARY

The authors determined active hydrogens of guanosine and yeast nucleic acid by the method proposed by Roger J. Williams which consists in dissolving the substances in deuterium oxide, evaporating to dryness and determining the increase in weight due to the replacement of active hydrogen by deuterium. The experiment showed that the number of active hydrogen of guanosine was 6, corresponding to the theoretical number, while the tetrasodium salt of the yeast nucleic acid was found to have 11–12 active hydrogens.

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The Journal of Biochemistry, Vol. 40, No. 1.

THE CHEMISTRY OF THE LIPIDS OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES

IV. DISTRIBUTION OF LIPID-HEXOSAMINE AND LIPID-HEMATAMINIC ACID IN THE RED BLOOD CORPUSCLES OF VARIOUS SPECIES OF ANIMALS

By TAMIO YAMAKAWA AND SHIZUE SUZUKI

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(Received for publication, September 16, 1952)

In the preceding paper (*1*), it was reported that a glycolipid, to which the name ' hematoside ' was assigned, was isolated from the equine blood stroma. Hematoside is composed of lignoceric acid, sphingosine, D-galactose and prehemataminic acid (probably N-acetylated) in the ratio 1:1:2:1. The purple Bial's test which characterizes the lipid, is responsible for the last component.

Hemataminic acid or methoxy-prehemataminic acid is considered by us (*2*) to be the same as neuraminic acid, which was first obtained in crystalline form by Prof. Klenk (*3*) as a component of ganglioside (*4*, *5*).

The presence of hexosamine in the lipid of bovine brain was already suggested by Blix (*6*), later he obtained chondrosamine in brain ganglioside and regarded Klenk's neuraminic acid as a degradation product by the isolation procedure (*7*).

Contrariwise, Klenk recently confirmed not only the presence of neuraminic acid but also that of chondrosamine in brain ganglioside (*8*).

Furthermore, he found chondrosamine in the glycolipid of human blood corpuscles (*9*).

Confirming this result, we obtained a glycolipid designated as ' globoside ' from human blood stroma (*10*). Globoside contained acetylchondrosamine besides fatty acids, sphingosine and D-galactose. It gave no purple color due to hemataminic acid with Bial's orcinol reagent. In this respect, it is entirely different from hematoside.

Thus, it becomes evident that though both belong to the mammals, human beings possess chondrosamine, whereas horses, hemataminic acid in their blood corpuscle glycolipid.

Therefore, it was deemed of the utmost importance to determine the distribution of these two substances in the red blood cells of various species of animals.

EXPERIMENTALS

The stroma samples were prepared as follows: Some 50 ml. of citrate-blood freshly taken was washed twice with physiological saline and hemolyzed with ten volumes of 0.3 per cent acetic acid solution. The precipitated stroma was spun down and washed with distilled water until the supernatant became colorless. The washed residue was dried from the frozen state. The yields of lyophilyzed stroma differed with species.

Lipid-Hexosamine: Ten to 80 mg. of the dried, powdered stroma was extracted with chloroform-methanol (1:3) for 2 hours in a micro extraction apparatus. The solution was transferred into a small Thunberg-tube and the solvent was cautiously evaporated. Hexosamine was determined with this dark-brown lipid extract by the procedure of Elson and Morgan (*11*) modified by Blix (*12*). The determination was made with Coleman electrophotometer at 540 mμ.

Lipid-Hemataminic Acid: The procedure of Klenk and Langerbeins (*13*) for the determination of neuraminic acid was slightly modified. At first, the light absorption of colored-complex of hemataminic acid with Bial's orcinol reagent was determined. An 1 ml. aliquot of aqueous solution containing 47.5 μg. of anhydrous hemataminic acid was pipetted into a small all-joint flask of 10 ml. content, 1 ml. of freshly prepared Bial's reagent was added and heated in an oil-bath at 142° for 5 minutes. After cooling, 5 ml. of pure isoamyl alcohol was added and the mixture was vigorously shaken in an ice-bath. After centrifuging for 5 minutes, the clear supernatant was removed with a pipette to a cuvette and the optical density was measured in a Beckman spectrophotometer Model DU. A reagent blank was treated in the same manner except that hemataminic acid was not added and no color developed. The absorption spectrum in the visual region was shown in Fig. 1.

The extinction-concentration curve of hemataminic acid using Pulfrich's photometer with a S 53 filter was in good agreement with that of neuraminic acid reported by Klenk and Langerbeins (*13*); it obeys Beer's law so far as the content was below about 40 μg. In this experiment, for the reason of conveniences, the quantitative determination of hemataminic acid was made with Coleman electrophotometer using 570 mμ.

Ten to 80 mg. of dried, powdered stroma was extracted in a small extraction apparatus with (i) acetone, (ii) ether, (iii) acetone for 15 minutes every time, then (iv) chloroform-methanol (1:3) for 30 minutes. The last extract was transferred into a flask of 10 ml. content, the solvent was carefully evaporated and the residue was dried in a desiccator. Dry ether was added, the ether was discarded and the determination was carried out according to the standard procedure described above.

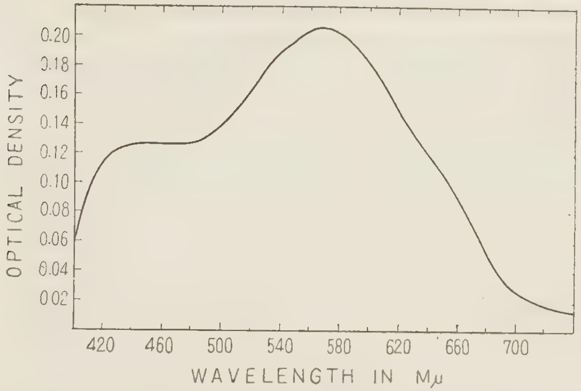

FIG. 1. Absorption spectrum of colored complex derived from hemataminic acid with Bial's orcinol reagent.

RESULTS AND DISCUSSION

The results of the analyses of lipid-hexosamine and lipid-hemataminic acid in the blood stroma of several mammalian along with an avian species are shown in the average values presented in Table I.

TABLE I

Distribution of Hexosamine and Hemataminic Acid in the Lipid of Red Blood Corpuscles

Species	Lipid-hexosamine	Lipid-hemataminic acid
	per cent of dry stroma	*per cent of dry stroma*
Human, Group O	0.18±0.03	non-detectable
„ , Group A	0.20±0.05	„ „
„ , Group B	0.20±0.05	„ „
Sheep	0.18±0.05	„ „
Goat	0.25±0.05	„ „
Hog	0.44±0.07	*
Beef	0.15±0.03	0.28±0.07
Rabbit	0.15*	0.24*
Dog	0.07±0.02	0.42±0.02
Horse	0.04±0.01	0.51±0.07
Chicken	0.02±0.01	non-detectable
Human Brain, Grey Matter	0.23±0.04	0.25±0.01
White Matter	0.09±0.03	non-detectable

* In these cases, yellowish or brownish coloration hindered the estimation.

From the results in the Table, it is easily found that four groups are divided on the ground of the contents of the two substances.

The blood stroma glycolipids of human, sheep, goat and hog possess hexosamine but no hemataminic acid, indicating they are, as it were, '*globoside type*.'

On the other hand, dog and horse have in its blood stroma lipid hemataminic acid and very little hexosamine, if any (*hematoside type*).

Bovine stroma possesses both components and chicken neither. The results obtained with rabbit's stroma were uncertain, because the coloration was somewhat brownish at the time of estimation.

In the grey matter of brain-cortex were found lipid-hexosamine along with the substance which gave purple color with Bial's reagent (hemataminic or neuraminic acid), but in much smaller amount in the white matter.

In view of the fact reported by Klenk that brain ganglioside contained both neuraminic acid and chondrosamine (*8*), it would be allowed to assume that ganglioside might probably be a mixture of hematoside and globoside.

The authors thank Prof. S. Akiya for his interest in this work.

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THE CONCENTRATIVE UPTAKE OF THIAMINE ALKYL DISULFIDES BY RABBIT OR CHICK BLOOD CELLS

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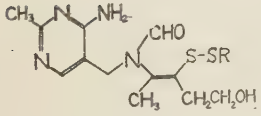
Fujiwara and Watanabe (*1*) have recently discovered that thiamine, added to the extract of garlic (*Allium sativum*), became a substance, which gave no thiochrome reaction, but which had anti-polyneurotic activity on thiamine-deficient rats. The product was named by them "allithiamine." Matsukawa and Yurugi (*2*) isolated it in crystalline form and established its chemical structure as 2-(2′-methyl-4′-amino-pyrimidyl-5′)-methyl-formamino-5-hydroxy-$\triangle^{2}$-pentenyl-(3) allyl disulfide. Further, its homologues were synthesized by Matsukawa and Kawasaki (*3*) that had other alkyl groups in place of allyl. The compounds were generically named by them "thiamine alkyl disulfides." (TRDs). These are listed as follows.



Allithiamine

R	Name	Abbrev.
methyl	Thiamine methyl disulfide	TMD
ethyl	Thiamine ethyl disulfide	TED
n-propyl	Thiamine *n*-propyl disulfide	TPD
allyl	Thiamine allyl disulfide	TAD
n-butyl	Thiamine *n*-butyl disulfide	TBD
i-butyl	Thiamine *i*-butyl disulfide	T *i*-BD
i-amyl	Thiamine *i*-amyl disulfide	T *i*-AmD
n-octyl	Thiamine *n*-octyl disulfide	TOD
benzyl	Thiamine benzyl disulfide	T BenzD

The authors have been in association with Matsukawa *et al.* in the biochemical studies on these disulfides, and by the assay using rice birds (*Uroloncha striata var. domestica*) have shown that all the compounds have almost the same activity to protect the development of thiamine-deficiency as thiamine (*4*). They are reducible to thiamine by the mammalian tissues. All these facts led us to conclude that they are not inferior to thiamine in their functional role *in vivo*. As they, unlike thiamine, are all soluble in most of organic solvents, they may be called " fat-soluble thiamine."

With regard to the absorbability of the disulfides, it was found by Fujiwara and Watanabe (*1*) that one of them, TAD, when orally administered to human being, was much more readily absorbed from the intestinal canal than thiamine, and as much excreted in the urine. This striking character appeared to be explained partly by the solubility in lipids. As to the mechanism of absorption of thiamine by animals, although many experiments had been carried out by other investigators (*5*, *6*, *7*) using the perfused gut or the isolated loop of intestine, yet the results obtained were inconsistent to each other. This was, probably due to the complexities of the systems used. Considering these facts, the authors decided to employ the blood cells of rabbit and of chick as our experimental material and intended to observe the uptake of these disulfides by them, with the anticipation that the disulfides may penetrate into them in a different manner from that of thiamine. The material used has the following advantages: (i) the stroma of the blood cell is extremely rich in lipids; (ii) the thiamine present in the normal blood is found, for the most part, to be concentrated within the cells and phosphorylated in the form of cocarboxylase (*8*, *9*); (iii) it is easy to determine the intra- and extra-cellular thiamine in the blood cell suspension. The present paper describes the results obtained.

MATERIALS AND METHODS

The blood of rabbits or chicks was collected by heart puncture using sodium citrate as anticoagulant. The blood cells were washed twice with Krebs-Ringer phosphate buffer (KRP-buffer) by centrifuging. In most case, the cells orginally contained in 3 ml. of the blood were thus resuspended in 10 ml. KRP-buffer (pH=7.0). A portion of 3 ml. of this suspension, mixed with a definite amount of $10^{-3}M$ thiamine or thiamine alkyl disulfide (TRD) solution (0.8 per cent NaCl), was shaken under air at 37° in Warburg's apparatus. With appropriate intervals, 1 ml. of the reaction mixture was pipetted out and, after being immediately cooled to 5°, was centrifuged to be separated into cells and medium. The cells were further washed with Ringer solution by centrifuge and then hemolyzed by adding an equal volume of distilled water. The medium and hemolysate, after being deproteinized by metaphosphoric acid, were analyzed for thiamine or TRD.

Thiamine was fluorometrically measured by the thiochrome method with cyanogen bromide as oxidizing agent, using a Klett fluorometer (*10*). For the determination of TRD, the sample was heated at 70° for 30 minutes after adding 30 mg. of cysteine hydrochloride. By this procedure TRD was quantitatively reduced to thiamine, which could be determined by the procedure for thiamine. All the experimental data were obtained at least in triplicates, of which the most typical one is described below.

RESULTS

Preliminary Experiment: When fresh rabbit blood cell suspensions were shaken in a medium initially containing $0.6 \times 10^{-3}M$ of TAD, the cells took up TAD so rapidly that the uptake nearly reached to the equilibrium in about 1 hour (Fig. 1, a). The decrease in amounts of total thiamine (thiamine+TAD) in the medium corresponded almost satisfactorily to the increase in that in the cell portion. TAD which remained in the medium was rapidly reduced to thiamine and the greater part of the intracellular TAD was also found to be free thiamine. When the cells which had fully taken up TAD was resuspended in KRP-buffer, the intracellular thiamine hardly went into the medium (Fig. 1, b). Contrary to TAD, thiamine was not taken up by blood cells at all, as shown in Fig. 1, a. Similar results were obtained in the case of thiamine disulfide and thiamine pyrophosphate ester.

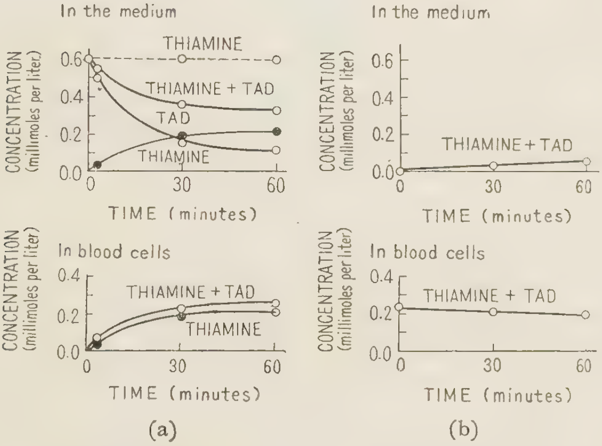


FIG. 1. The concentration of thiamine and TAD found in the medium and the rabbit blood cells (at 37°).
(a) Continous line: suspended with $0.6 \times 10^{-3} M$ TAD. Broken line: suspended with $0.6 \times 10^{-3} M$ thiamine.
(b) Resuspended in the TAD-free medium after 1st run with TAD.

The Concentrative Uptake of Thiamine Methyl Disulfide by Chick Blood Cells: It was investigated whether the concentrative uptake of

TRDs by chick blood cells would depend on the process of solution in, and diffusion through, the lipid layer of blood cell membranes. If the slowest step in the penetration is the process of diffusion through a lipid layer, then the initial rate of transport is given by the Fick's law:

$$dQ = RT\frac{dc}{dx}K$$

where dQ is the amount diffusing in time dt, dc/dx concentration gradient, K the constant characteristic to the molecule and the membrane. The concentration gradient will in turn depend upon the partition coefficient of a solute between cell membrane material and external medium (*11*).

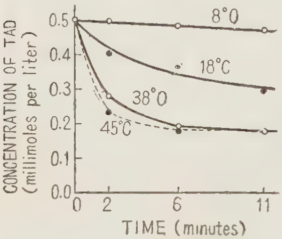


FIG. 2. The effect of temperature upon the uptake of TMD by chicken blood cells.

The initial rate of decrease in concentration of TMD in the medium was dependent on temperature (Fig. 2), the transfer nearly stopping below 8°. It was, however, not exactly proportional to the absolute temperature.

In order to study the effect of the concentration gradient, the chick blood cell suspensions were incubated at 38° in the initial TMD concentration of 0.3×10^{-3}, 0.6×10^{-3} and 0.9×10^{-3} *M*, respectively. The decreases in the extracellular TMD concentrations are indicated in Table I. The amounts of TMD uptake depend upon the initial TMD levels. Namely, the ratio of the decrease of TMD to its initial conccentration was almost constant for different initial concentrations. This fact indicates that the process has close relations to the concentration gradient.

TABLE I

The Relation between Concentration Gradient and the Uptake of TMD

Initial concentration of TMD in medium (A)	The amounts of TMD taken up during 2 minutes	6 minutes (B)	Ratio B/A
millimoles per liter	*micromoles per ml.*	*micromoles per ml.*	
0.300	0.022	0.048	0.160
0.600	0.050	0.104	0.173
0.900	0.075	0.175	0.194

At 38°, chick blood cells.

Concerning the effect of pH upon the uptake of TMD by chick blood cells, the experiments were carried out at pH 7.4, 6.8 and 5.8. As shown in Fig. 3, the rate of transfer diminished in the acidic pH range. TMD was found to have the pKa value of 5.59 (at 25°), which is attributed to the dissociation of 4-amino group of its pyrimidine ring. So, the n-butanol/0.1 *M* citrate-Na_2HPO_4-buffer partition coefficients of TMD were measured at the pH of 7.6, 5.6 and 3.6 at 25°. The values found were 13.2, 8.5 and 1.2, respectively (Fig. 3). If the theoretical pH is plotted against the percentage of the unionized form of TMD, the close agreement can be seen among the three curves with each other. From this result it was evident that the uptake was profoundly related to the partition coefficient and that only unionized molecules of TMD could penetrate into the cells.

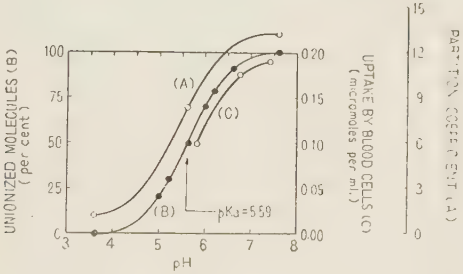


FIG. 3. The effect of pH upon the rate of uptake of TMD, partition coefficient and percentage ionization of TMD.

(A): Partition coefficient of TMD between *n*-butanol and 0.2 *M* phosphate-0.1 *M* citrate buffer, at 25°. (B): The theoretical pH-ionization curve of TMD, theoretically derived from its pKa. (C): The amount of TMD taken up by chicken blood cells during 25 minutes, at 20°.

It is well-known that the permeability of cell membrane against an electrolyte is fairly influenced by the ionic constituents of the medium. Therefore, the experiments were carried out using the isotonic $CaCl_2$, KCl and NaCl solutions instead of KRP-buffer. It was, however, observed that the alteration in the ionic compositions gave no appreciable effect.

From the above-mentioned results it is conclude that the penetration of TMD and TAD into the blood cells proceeds, at least qualitatively, in accordance with the Fick's law while thiamine can not be taken up by such a simple diffusion process.

The Comparative Study on the Uptake among the TRD Homologues: From the above-mentioned data concerning the effect of pH on the uptake, the unionized form of TMD with greater partition coefficients than the ionized was found to be much more readily taken up by blood cells. Therefore, the authors intended to compare the absorbabilities among the *n*-alkyl disulfide homologues. At first the authors measured benzene/0.1 *M* phosphate buffer (pH 6.6)—and *n*-butanol/0.1 *M* phosphate

Table II

Partition Coefficient and pKa of Thiamine and Its Alkyl Disulfides

Compounds	Partition coefficient*		pKa*
	Benzene/Buffer**	*n*-Butanol/Buffer**	
Thiamine HCl	<0.0001	0.044±0.007	4.70
Thiamine disulfide	<0.0001	3.2 ±1.2	5.30
Thiamine meteyl disulfide	0.083±0.035	12	5.59
Thiamine ethyl disulfide	0.21 ±0.03	24	—
Thiamine *n*-propyl disulfide	0.77 ±0.22	58	5.52
Thiamine allyl disulfide	0.44 ±0.11	40	5.65
Thiamine *n*-butyl disulfide	2.7 ±0.2	125	—
Thiamine *i*-butyl disulfide	2.4 ±0.3	120	—
Thiamine *i*-amyl disulfide	8.6	260	5.58
Thiamine *n*-octyl disulfide	600 ±380	3500	—
Thiamine benzyl disulfide	4.9	120	—
Thiamine methyl disulfide	*n*-Butanol/0.5*M* Phosphate buffer***	16.0	
Thiamine methyl disulfide	*n*-Butanol/0.05*M* ” ”	11.0	

* At 25°, $\bar{X}\pm t.05\times S\bar{x}$. ** 0.1 *M* Phosphate buffer, pH 6.62. *** pH 6.60

Table III

The Uptake of Thiamine n-Alkyl Disulfides by Chick Blood Cells

Compounds	Millimolar concentration (medium)* t=0	t=6 minutes	Uptake during 6 minutes
			micromoles per ml.
Thiamine methyl disulfide	0.500±0.016	0.364±0.008	0.136
Thiamine ethyl disulfide	0.500±0.010	0.344±0.006	0.156
Thiamine *n*-propyl disulfide	0.500±0.010	0.227±0.003	0.273
Thiamine *n*-butyl disulfide	0.500±0.018	0.180±0.004	0.320

* At 18°. $\bar{X}\pm t.05\times S\bar{x}$

(pH 6.6)—partition coefficient of TRDs at 25°. As shown in Table II, the logarithm of both the partition coefficients bears a linear relationship to the number of carbon atoms in the alkyl side chain, which is well in accordance with the Traube's rule (Fig. 4). Then, the authors studied on the penetration of thiamine *n*-alkyl disulfides into chick blood cells at 18°. Table III indicates that the longer the alkyl side chain is, the greater the rate of uptake becomes. The initial rate of uptake depends on the partition coefficient, therefore, on lipid-solubility.

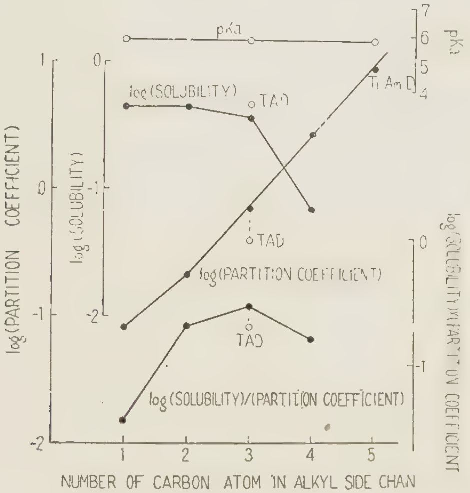


Fig. 4. Some physico-chemical properties of thiamine alkyl disulfides.

Solubility: log (g/100 ml. H_2O), at 25°.

Partition coefficient: log. (partition coefficient), benzol/0.1 *M* phosphate buffer, pH 6.62, at 25°.

pKa: at 25°.

DISCUSSION

The above experimental results indicate that TRDs can be concentrated in much higher level than the physiological level of thiamine in the normal blood cells and that the concentrative uptake depends mainly on the simple diffusion due to their lipid-solubility. In order to draw out a clue in respect of their absorbabilities *in vivo*, the authors glanced at the variation of the physico-chemical factors, such as pKa, partition coefficient and solubility with the increase of alkyl side chain length. As illustrated in Fig. 4, the pKa values are always found in the vicinity of 5.58 among the homologues. That is, in the physiological pH range the greater part of the molecules of each homologue exists in a completely unionized state. Therefore, pKa is not an important factor in our comparative viewpoint. The partition coefficient increases with the increase in size of alkyl chain. Namely, the longer the alkyl side chain is, the higher the ratio will be held between the cellular and extracellular concentrations. On the other hand, increase in carbon number of the side chain, lowered the water-solubility of the series, to such an extent that TOD, for example, is actually insoluble. The incompatible responses between partition coefficient and solubility to the elongation of the side chain are enough to anticipate the possibility that among the homologues there exists a one of the most effective absorbability *in vivo*. With such an anticipation we plotted the logarithm of the products of partition-coefficient and solubility against the carbon number and obtained the convex curve, whose peak was found in the position of C_3 (Fig. 4).

Under our experimental conditions, only TRDs were observed to penetrate into blood cells, while neither thiamine, thiamine disulfide nor thiamine pyrophosphate was taken up in appreciable amounts, possibly due to their extremely low partition coefficient (lipid/water). It is, however, significant to note again that thiamine contained in the normal whole blood of higher animals is reported to exist mainly within leucocytes and erythrocytes, of which the greater part is found to be phosphorylated in the form of its pyrophosphate ester (*9*, *10*). This fact suggests that thiamine, although extremely fat-insoluble, might possibly be concentrated in blood cells by a particular mechanism such as so-called " active diffusion " process coupled with energy-provision and phosphorylation. If the lipid-insoluble compound such as thiamine could penetrate into blood cells without any specific mechanism, the process would be inconsistent with the second law of thermodynamics. It was, however, observed in our further studies that the uptake of thiamine was not carried out even in the presence of glucose or adenosine triphosphate, the addition of which remarkably stimulated the respiration By use of the nucleated blood cells of chick having more active metabolic function than mammalian blood cells, the concentrative uptake could not be observed. The uptake might have been too small to be appreciated by our determination method, presumably due to the physiologically too high concentrations of thiamine used, even if it had occurred by a normal mechanism. For the purpose of elucidating the mechanism, further experiments would be desirable in much lower range of thiamine concentration.

Fujiwara *et al.* (*1*) previously reported that crude allithiamine was reduced to thiamine by liver slices. Their findings were confirmed by the experiments on the reduction of pure allithiamine by the homogenates of rabbit tissues. It is known that liver, spleen and blood cell are the most active tissues, while kidney, brain, skeletal muscle and blood plasma exert much less activity. There was, however, shown no optimal pH in the pH-activity relationship, where the activity greatly diminished below pH 5.0 and went up rapidly above pH 10. The boiled extracts of the homogenates (at 100° for 15 minutes) still preserved completely the original reducing activities. Therefore, the question will remain unsolved whether the reduction, like that of the oxidized form of glutathione by glutathione reductase (Rall and Lehninger (*12*), is enzymatic. The detail in this point will be reported later.

SUMMARY

Using the suspension of rabbit or chick blood cells, the concentrative uptake of thiamine and thiamine alkyl disulfides by the cells was observed.

1. Neither thiamine, thiamine disulfide nor thiamine pyrophosphate was taken up by the cells at all. The addition of glucose or adenosine triphosphate did not favour the uptake of them.

2. Thiamine alkyl disulfides tested were all readily taken up by the blood cells, the rate of uptake depending upon temperature, concentration gradient and pH in the medium. From the effect of pH on the dissociation and partition coefficient, only the unionized form of thiamine methyl disulfide seemed to be transferred by the simple diffusion process.

3. The partition coefficients of the thiamine alkyl disulfide homologues beared logarithmically a linear relationship to the mumber of carbon atoms in the alkyl side chain. Among the homologues the rate of penetration depends upon the partition coefficient.

4. From all these results, it was concluded that, unlike thiamine, thiamine alkyl disulfides were taken up by the blood cells according to the process of solution in, and diffusion through, the lipid membrane.

Acknowledgment. All samples of thiamine derivatives were kindly offered by Dr. Matsukawa. The technical assistance of Miss M. Kurihara is gratefully acknowledged. The authors are deeply indebted to Mr. Asahi for measuring pK, and Mr. Terao for measuring the solubility. The authers' thanks are also due to Dr. Kuwada, Director of our Research Laboratory and to Dr. Ninomiya, our previous chief, for their continued encouragements.

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UPON THE LINKAGE OF ARGININE IN PROTEIN MOLECULE

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It is a matter of dispute whether NH_2- radical of guanidine group of arginine in protein molecule is free or not. Now, for the solution of this problem the author's arginine reaction (1925) (*1*) may give the clue, as this reaction is only positive when the guanidine group is free. Apparently the arginine reaction applied directly to protein molecule does not give the equal value to that of its hydrolysate. This fact shows that the guanidine group of arginine in protein molecule is to a part in a combined state. In this case the argument, that arginine in a combined state as in protein molecule may give weaker colour reaction even when its guanidine group is free, can not be tenable, as shown later in respect of peptone. It was intended in this experiment to know how far arginine or guanidine group is liberated by partial acid hydrolysis to give full colour reaction and thus to elucidate the attitude of arginine in the structure of protein molecule.

I. Arginine Reaction of Protein and of Its Acid Hydrolysate—For the determination of arginine the author's new method (*2*) was used. This determination was carried out on the dilute solution (1:20000) of protein dissolved by aid of small amount of alkali. The colour developed is somewhat tinged with red compared with that of free arginine. The results obtained with a filter, S_{50} of Pulfrich's photometer on various proteins and their complete hydrolysates by hydrochloric acid are showed in Table I.

The intensity of arginine reaction of most proteins studied was nearly one third to that of hydrolysates except peptone which showed more than a half.

II. Arginine Reaction of Partial Acid-Hydrolysate of Protein—Assuming that arginine which does not enter into this reaction, is due to the blockade of guanidine group, the following experiments were carried out in order to know how far such blockade will be released by acid hydrolysis of various grades to give positive reaction.

TABLE I

Arginine Value of Protein and its Hydrolysate

Sort of protein	A Before hydrolysis	B After hydrolysis	$\frac{A\times 100}{B}$
	γ/*ml.*	γ/*ml.*	
Arachin	2.45	6.50	37.8
Caseinogen	0.70	1.87	37.4
Gelatin	1.80	3.90	46.2
Gliadin	0.50	1.35	37.0
Oryzenin	1.68	5.00	33.6
Peptone (Witte)	1.94	3.37	57.6

Experiment 1. A portion of 100 mg. of protein was taken in a test tube provided with glass-rod and glass-cap*, mixed with hydrochloric acid of known concentration and heated for 3 hours in a boiling water-bath under occasional stirring or shaking, cooled, made alkaline with sodium hydroxide and filtered. With this filtrate the following determinations were carried out:

Determination of Arginine in Hydrolysates—The hydrolysates were highly diluted to the extent of one part of protein in 20000–40000 parts of solution, thus avoiding the possible effect of coexisting substances. The determination was carried out against the standard arginine solution containing 1–2 γ in 1 ml., using an optical cell of 20–30 mm. The results are indicated in percentage of the value obtained on complete hydrolysates.

Determination of Free Arginine and Arginine Peptide Liberated by Hydrolysis—This method is based on the fact that a mixture which holds arginine solution (10 mg. per cent) and phosphotungstic reagent (3 g. in 100 ml. of 5 per cent HCl) in equal volume is almost clear at 20°, showing that under this condition free arginine molecule does not precipitate. To a dilute hydrolysate (0.02 per cent) to be tested equal volume of phosphotungstic reagent was added and filtrated after allowing to stand for a day. A portion of 5 ml. of this filtrate was transferred to a volumetric flask turned to weak alkaline reaction and diluted to the 100 ml. mark with water. The solution, if not clear, should be filtered after adding a small amount of caolin (0.05–0.1 g.).

Determination of Amino-N—Amino-N was determined by the Van Slyke's method. Results are indicated as the percentage of the value of complete hydrolysate.

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* See this *Journal*, Vol. **37**, p. 236 (*2*).

As shown in Table II, the arginine reaction of hydrolysates showed a distinct increase inspite of mild hydrolysis in relatively short time. In the case of 0.3 *N* HCl the arginine reaction of oryzenin increased 24.8 per cent against original protein, but the value of free arginine was very

TABLE II

Increase in Arginine value by Acid Hydrolysis

(Time of heating, 3 hours. Temporature, 100°)

	Concentration of HCl	Arginine value		Amino-N
		Hydrolysate	Free arginine	
	N	*per cent*	*per cent*	*per cent*
Oryzenin	0	33.6	—	—
	0.1	44.4	—	7.2
	0.2	53.0	—	11.2
	0.3	58.4	4.4	14.7
	0.5	64.2	8.0	20.0
	1.0	79.6	19.5	32.4
	1.5	90.0	28.6	40.0
	2.0	95.4	34.8	44.0
	3.0	99.0	50.1	55.0
Arachin	0	37.8	—	—
	0.1	43.6	—	9.5
	0.2	56.7	—	14.7
	0.3	60.0	6.5	16.8
	0.5	66.5	10.5	22.0
	1.0	82.0	26.6	33.2
	1.5	90.5	31.6	45.7
	2.0	96.5	37.0	54.7
	3.0	99.5	44.2	57.4
Gelatin	0	46.2	—	—
	0.1	51.0	—	15.7
	0.2	69.1	—	22.5
	0.3	78.3	8.9	25.9
	0.5	81.5	26.0	38.0
	1.0	89.3	38.1	48.2
	1.5	94.6	40.9	60.2
	2.0	97.0	44.0	66.6
	3.0	99.0	51.8	72.2
Globulin	0	30.7	—	—
	0.1	43.7	—	8.1
	0.2	51.7	—	15.0
	0.3	55.5	7.3	17.5
	0.5	67.5	10.5	22.0
	1.0	80.3	22.6	33.0
	1.5	92.0	32.7	43.3
	2.0	99.0	44.1	48.6
	3.0	99.0	53.1	56.3

small. The liberation of arginine molecule increased at a conspicuous rate in the case over 0.3 *N* HCl. For example, free arginine value of oryzenin in the case of 1 *N* and ~~2~~ 3 *N* HCl were 19.5 and 50.1 per cent, respectively.

Further, Table II shows that when protein was heated with 3 *N* HCl for three hours, nearly full arginine values were obtained in all kinds of protein, though the hydrolysis was not complete. In this case the increase of amino-N was 55–57 per cent exept gelatin (72.2 per cent).

TABLE III

Arginine Values of Precipitate and Filtrate of the Hydrolysate by 0.1 *N HCl at Various Duration of Heating*

Protein		Duration of heating in hours			
		1	2	5	10
Oryzenin	Hydrolysate	(42)	(44)	(45)	(55)
	Precipitate	71.4	52.0	45.7	17.5
	Filtrate	28.6	48.0	54.3	82.5
Globulin	Hydrolysate	(42)	(43)	(44)	(51)
	Precipitate	69.6	54.4	35.9	12.2
	Filtrate	30.4	45.6	64.1	87.~~6~~8
Arachin	Hydrolysate	(41)	(42)	(43)	(50)
	Precipitate	62.3	46.0	32.2	8.7
	Filtrate	37.7	54.0	67.8	91.3

After treating with 0.1 *N* HCl for various length of time, the hydrolysate was neutralyzed, upon which an insoluble acid protein came out and was separated by centrifuge. On the precipitate and the filtrate the arginine reaction was carried out. The results are given in Table III.

As shown in Table III the arginine value of the hydrolysate as a whole did not vary much in the course of heating for 5 hours, though the amount of precipitate decreased considerably.

III. Arginine Reaction of Peptone—In the foregoing experiment the precipitate, which corresponds to a socalled acid protein obtained from mild acid hydrolysate of protein was proved to give nearly the same colour intensity of arginine reaction as that of original protein. But this is not the case with peptone, which was obtained by treating protein with more concentrated hydrochloric acid followed by precipitating with tannic acid. Such a preparation of peptone from any sorts of protein gives nearly 100 per cent arginine value. This fact is very interesting because it offers evidence that arginine molecule can give full reaction even in the combined state when its guanidine group is free.

DISCUSSION

Since the guanidine group of arginine is strongly basic and its ionisation more pronounced than that of the amino group of amino acid, it is quite provable that the guanidine group of arginine molecule reacts with any acidic group of other amino acid in protein molecule. In such a state arginine in protein molecule does not give full colour reaction. By treatment with quite dilute acid this reaction becomes more intensive. This means that the linkage is relatively weak and can be splitted by mild treatment. Perhaps such a linkage may bring about interpeptide chain, thus establishing special shape of protein molecule.

SUMMARY

1. Nearly all sorts of protein give arginine value about one third to that of its hydrolysate. This means that two third of arginine molecules in protein enters into combination of its guanidine group.

2. The guanidine linkage of arginine in protein molecule is easily splitted by mild treatment of acid.

3. The arginine molecule bound in peptide chain can give the full colour reaction, if its guanidine group is free.

In conclusion the author wishes to express my hearty thanks to Prof. Emeer. S. Kakiuchi and Prof. K. Kodama for their kind advice.

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MECHANISM OF MUSCULAR CONTRACTION. I. INTERACTIONS BETWEEN ACTOMYOSIN AND ADENOSINE TRIPHOSPHATE‡

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INTRODUCTION

It is well known that upon the addition of adenosine triphosphate (ATP) to actomyosin solution, the viscosity (*1*), the double refraction of flow (*2*), the light-scattering (*3*) *etc.* of actomyosin solution are changed while ATP is split to adenosine diphosphate (ADP) and inorganic orthophosphate (P) (*4*).

In other words, the deformation of actomyosin particles and coincidently the adenosine-triphosphatase (ATPase) action are then observed.

These two reactions being thought to represent fundamental mechanisms of muscular contraction have so far been studied by many investigators but their mechanisms have been poorly clarified owing to the difficulty of the exact measurements of these phenomena.

The present writers have estimated the inorganic orthophosphate split in consequence of ATPase action using a precise electrophotocolorimeter and they have, on the other hand, investigated the deformation of actomyosin particles by means of measurements of the light-scattering. On the basis of these experiments, the mechanisms of these two phenomena were clarified respectively and the interrelation of them was established; further, it became possible to interpret many facts that have already been learned about muscular contraction.

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‡ The contents of this paper were presented at the 4th (August 1951, Tōkyō) and the 5th meeting of the Symposia on Enzyme Chemistry (July 1952, Ōsaka), and the 5th annual meeting of the Chemical Society of Japan (April 1952, Tōkyō). Parts of them have already been published in *Nature* 169, 112 (1952) and in *Symp. Enz. Chem.*, (in Japanese), 7, 46, (1952).

PREPARATION OF MATERIALS

Preparation of ATP—Fresh muscle cut from rabbits anaesthetized rapidly (within about 2 or 3 minutes) with chloroform was dried by the acetone treatment. This acetone dried muscle was extracted with hot water. The extracted solution was brought to 0.3 per cent by volume acetic acid solution by the addition of glacial acetic acid and the precipitates formed were removed. ATP was isolated from the resulting clear fluid as Hg-salt and then as Ba_2-salt according to Kerr's method (*5*).

The yield of the Ba_2-salt is about 2 gram per Kg of fresh rabbit muscle and its purity is 75—80 per cent. But the raito of P:N is 1:1.30 and the ratio of the quantity of phosphorous split by 7 minutes-hydrolysis in N-HCl, at 100° (7′ P) to that of phosphorous liberated in the case of hydrolysis with H_2SO_4-H_2O_2 (Total P) is about 2:3 in our ATP preparations. Therefore, its impurities should be inorganic Ba-salts and were probably removed as Ba_2SO_4 in the case of the converting Ba_2-ATP into a neutral K-ATP which was used for our experiments.

Preparation of "Purified Myosin B"—The minced striated muscle of rabbit hind leg was suspended in ice-cold Weber's solution (0.6 *M* KCl, 0.04 *M* $NaHCO_3$, 0.01 *M* Na_2CO_3). The suspension was kept in the ice box (0—5°) for about 24 hours. After this time, the suspension was centrifuged.

The resulting clear supernatant fluid was adjusted to pH 6.5 by adding dilute acetic acid and was diluted with ice-cold distilled water, about 5—6 volumes being added for every volume of the supernatant. The flocculent precipitate formed was allowed to settle overnight in a cold room and then the clear supernantant fluid was siphoned off. The settled precipitates were centrifuged, washed twice with ice-cold distilled water. The "Myosin B" precipitate was dissolved in 0.5 *M* KCl solution and the insoluble matter was removed by centrifugation.

The myosin B solution was again diluted with ice-cold distilled water, three volumes being added at this time. The precipitate was again centrifuged, washed with cold distilled water and redissolved in KCl. "Myosin B" was purified by two or three times repeating this procedure.

Finally, the myosin B was obtained as a solution containing 0.5 *M* KCl.

ATPASE ACTION

Methods of Measuring Enzyme Activity—The enzyme reaction was started by adding 0.5 ml myosin B solution (1—3 mg. protein per ml.) to the mixed solution which is composed of 1.5 ml buffer solution (0.1 *M* glycine—0.1 *M* KOH, 0.1 *M* veronal-acetate, or 0.1 *M* citrate), 0.5 ml. K-ATP solution, 0.25 ml. $CaCl_2$aq. or H_2O and 0.25 ml. $MgCl_2$-aq. or H_2O (the potassium content in this reaction mixture is about 0.15—0.18 *M*; when the concentration of potassium was desired to be 0.4—0.5 *M*, concentrated KClaq. was used instead of H_2O). After certain times (usually 1,2 and 3 minutes), the reaction was stopped by adding 1.0 ml. of 10 per cent CCl_3COOH or 20 per cent $HClO_4$.

This digest was filtered through the filter paper and an aliquot of this filtrate (1 or 2 ml.) was then analyzed for free ortho-phosphate liberated according to Briggs' method (*6*) or Youngburg-Youngburg's mothod (*7*). The analysis was carried out colorimetrically using an electrophotometer. The electrophoto-colorimeter was constructed according to Müller *et al.*(*8*) and was schematically shown in Fig. 1. In this apparatus the electrophoto-tube PG-50-G (Matsuda) and the filter VR-2 (Matsuda) were used.

Then, the concentration of ATP was calculated from the quantity of 7′ P (acid labile phosphate), presuming 7′ P to be two-thirds of Total P in ATP. The content of protein was determined by the Kjeldahl method using a factor of 6.25 and pH values reported were measured with a Beckman pH-meter (G-type).

Results and Discussion—The purified myosin B used splits off only one phosphate residue from one molecule of ATP.

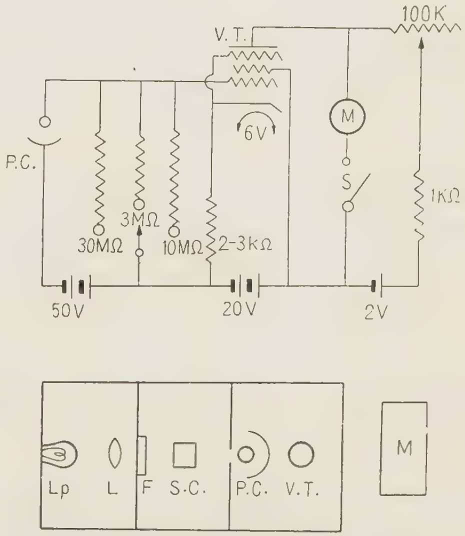

FIG. 1. Electrophoto-colorimeter. P.C.: electrophoto tube PG-50-G, V.T.: vacuum tube 6D6, M: μ-ampere meter 300 μA, LP: lamp 6V 10W, L: lens, F: filter VR-2 S.C.: sample vessel, S: switch.

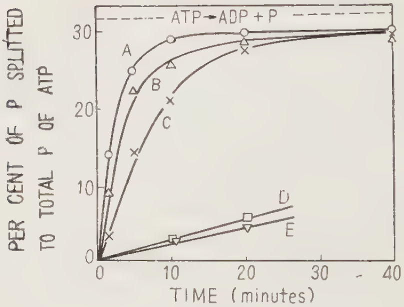

FIG. 2. ATPase action of purified myosin B. 10°, [ATP] 5×10^{-3} *mole/lit.* [$CaCl_2$] 5×10^{-3} *mole/lit.* and [$MgCl_2$] 5×10^{-5} *mole/lit.* A: glycine buffer at pH 9.2 (Ca^{2+} addition), B: veronal-acetate buffer at pH 9.2 (Ca^{2+} addition), C: veronal-acetate buffer at pH 6.3 (Ca^{2+} addition), D: glycine buffer at pH 9.2 (Ca^{2+} and Mg^{2+} additions), E: glycine buffer at pH 9.2 (Control)

Its ATPase activity is strongly activated by Ca^{2+} ion (without addition of Ca^{2+}, its activity is very slight and therefore all enzymatic experiments reported below were made in the presence of $CaCl_2$) and this activating effect is inhibited by the addition of $MgCl_2$ thereto (see Fig. 2).

Estimation of Michaelis Constant: Michaelis constant (K_m) of ATPase has not yet been determined on account of the difficulty of measuring the micro amount of phosphorous. We made an attempt to estimate K_m using Youngburg's method (#). Two examples from among the results obtained are shown in Fig. 3a–b. Our results

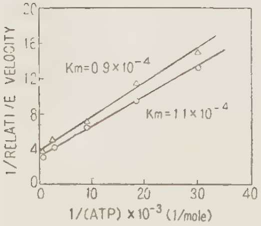

FIG. 3a. Initial velocity-substrate concentration relationship in the presence of 1.6×10^{-2} *mole/lit.* $CaCl_2$ (circle) or $CaCl_2$ plus 2.5×10^{-5} *mole/lit.* $MgCl_2$ (triangle). Temperature at 6°. Veronal-acetate buffer at pH 6.3.

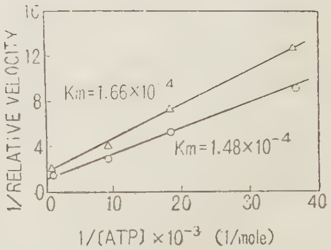

FIG. 3b. Initial velocity-substrate concentration relationship in the presence of 1.6×10^{-2} *mole/lit.* $CaCl_2$ (circle) or $CaCl_2$ plus 2.5×10^{-5} *mole/lit.* $MgCl_2$ (triangle). Temperature at 11°. Glycine buffer at pH 9.3.

# Youngburg's method is about four times as sensitive as Fiske-Subbarow's method employed usually.

are not satisfactorily exact but gave roughly a straight line when a reciprocal of initial velocity was plotted against the reciprocal of substrate concentration.

The Michaelis constants obtained in the presence of Ca^{2+} ion and Mg^{2+} ion are given in Table 1.

TABLE I

Michaelis Constant of ATPase

(10°, veronal-acetate buffer, [K] 1.8×10^{-1} *mole/lit.* $[CaCl_2]$ 1×10^{-2} *mole/lit.* $[MgCl_2]$ 1×10^{-4} *mole/lit.*)

pH	K_m (*mole/lit.*) in the presence of	
	Ca	Ca+Mg
9.0	$1.0\sim1.9\times10^{-4}$	$0.7\sim1.9\times10^{-4}$
6.3	$1.0\sim1.9\times10^{-4}$	$0.8\sim1.4\times10^{-4}$

These experiments suggest that the ATPase action may be formulated as follows;

$$M^* + S \xrightarrow[k_1^*]{} M^*S \qquad (1^*)$$

$$M^*\ S \xrightarrow[k_2^*]{} M^* + P \qquad (2^*)$$

and k_1^*/k_2^* is about 1.5×10^{-4} *mole/lit.* (♯) where M and S represent respectively the enzymatic unit of actomyosin and an ATP molecule and then a symbol * affixed upon M stands for the deformated state in which actomyosin during the enzymatic reaction is present.

Now, the value of k_2^* referred to one mole myosin in the presence of Ca^{2+} ion, at pH 6.,3 and 21° is 44 $sec.^{-1}$, hence (by neglecting the temperature dependence of K_m) $k_1^*=30\times10^4$ *lit./mole.sec.* As one mole of myosin contains six units (♯♯), the values per unit are calculated to be;

♯ According to J.J. Blum (personal communication), a similar value of K_m was obtained by Quellet using Fisk-Sabbarow's method. The reverse reaction of the reaction (1*) is neglected by us on the basis of the analogy about the results of the light-scattering experiments.

♯♯ The unit is 140,000 g. myosin or the quantity of actomyosin which contains 140,000 g. myosin. This quantity was chosen for such reason as will be reported in p. 45.

$${}_{Ca}k_1^* = 5 \times 10^4 \ \textit{lit./mole.sec.} \qquad (\#),$$
$${}_{Ca}k_2^* = 7 \ \textit{sec.}^{-1}$$

It is further obvious from Table I that the addition of Mg^{2+} ion does not affect the K_m value. Most of experiments described below were conducted under such a condition that the ATP concentration is sufficiently large and so the reaction (2*) is the rate determining step.

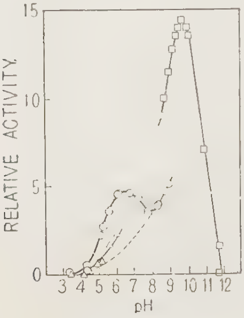

FIG. 4. Effect of pH on ATPase activity in the presence of Ca^{+2}. 10°, [ATP] 6×10^{-4} *mole/lit.*, $[CaCl_2] \times 10^{-2}$ *mole/lit.*

Effect of pH: As indicated in Fig. 4, the pH-activity curve shows two optima at around pH 6.3 and 9.7.

This fact may be interpreted as follows: The optimum at pH 9.7 is probably due to the fact that the acidic and basic components of the enzyme are inactive as in most enzymes. Then, the optimum at pH 6.4 may be due to such a condition that the complex of enzyme-ATP undissociated in its phosphate residue (M*S) is more reactive than that of enzyme-ATP dissociated (M^*S^-). That is, by assuming that the dissociation constant for the reaction $M^*S^- + H^+ \rightleftarrows M^*S$ is $10^{-6.5}$ (*9*) just as that for the reaction $S^- + H^+ \rightleftarrows S$ and that the velocity of M*S splitting is five times faster than that of M^*S^- splitting and that if not so the pH-activity curve should be the dotted line in Fig. 4, the pH-activity curve denoted by a big line which is in good agreement with the observed one, is obtainable.

The values of activation energy of the ATPase action at various pH are given in Table II.

Inhibition by Mg^{2+} Ion: At the concentration of K^+ ion used in our experiments (0.16 *M* or so and about 0.48 *M*), ATPase activity of purified myosin B is inhibited by Mg^{2+} ion even if used alone while it is activated by Ca^{2+} ion (Table III) (##). This activating effect of Ca^{2+} ion is competitive with the inhibiting effect of Mg^{2+} ion (Fig. 5).

${}_{Ca}k_1$ is representative for the velocity constant of the reaction (1*) in the presence of Ca^{2+} ion and the same rule applies correspondingly to the following parts.

Further, ${}_{Ca}k_2^*$ at pH 7.0 (glycine buffer) and at 37° is about 42 $\textit{sec.}^{-1}$

## It has been reported that the behaviour of the glycerinized muscle-ATPase (10), unpurified myosin A-ATPase and unpurified myosin B-ATPase (42) towards

Table II

Activation Energy (ΔH^* Kcal) of ATPase Action
([K] 1.7×10^{-1} *mole/lit.*, [ATP] 1×10^{-5} *mole/lit.*)

Buffer	pH	$CaCl_2$	$MgCl_2$	ΔH^*
		mole per liter	*mole per liter*	*Kcal.*
Glycine	9.5	1.3×10^{-2}	0	27
Veronal-acetate	9.4	1.0×10^{-2}	0	25
"	6.9	"	1×10^{-4}	22
"	8.0	"	0	15
"	7.1	"	0	10
"	6.8	"	0	16
"	6.8	"	1×10^{-4}	9.3
"	6.5	1.3×10^{-2}	0	19
"	5.9	1.0×10^{-2}	0	21

Table III

Effect of Ca^{2+} aud Mg^{2+} on ATPase Activity
(10° glycine buffer (pH 9.2), [ATP] 1×10^{-3} *mole/lit.*, [K] 1.5×10^{-1} *mole/lit.*)

$CaCl_2$ (*mole/lit.*)	Relative Activity*	$MgCl_2$	Relative Activity*
		mole per liter	
4×10^{-2}	7.2^{5}	20×10^{-4}	0.2
2×10^{-2}	9.5^{5}	10×10^{-4}	0.2
1×10^{-2}	9.2^{5}	5×10^{-4}	0.3
0.5×10^{-2}	7.0	1×10^{-4}	0.3
0.1×10^{-2}	4.1	0.5×10^{-4}	0.7^{5}
		0.1×10^{-4}	1.0

$$*\text{Relativea ctivity} = \frac{\text{Initial velocity of ATPase action in the presence of } Ca^{2+} \text{ or } Mg^{2+}}{\text{Initial velocity of ATPase action in the absence of } Ca^{2+} \text{ or } Mg^{2+} \text{ (control)}}$$

Ca^{2+} and Mg^{2+} were different from that of the purified myosin-ATPase but this difference may come from the situation that Mg-activated ATPase, myokinase *etc.* are also present in these preparations.

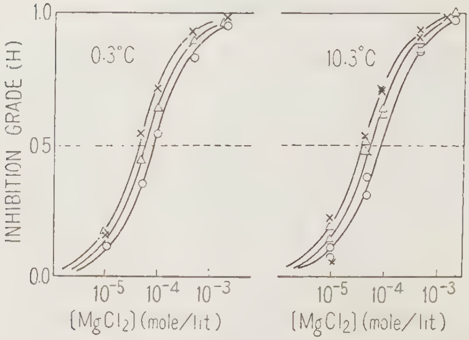

FIG. 5. Mg- inhibition of ATPase. glycine buffer pH 9.3, ATP 1×10^{-3} *mole/lit.* $[CaCl_2]$ 2×10^{-2} *mole/lit.* (○), 1×10^{-2} *mole/lit.* (Δ) and 0.5×10^{-2} *mole/lit.* (×).

When inhibition grade H is defined as:

$$H = 1 - V_{Mg} / V_{Ca}$$

where V_{Ca} is the initial velocity in the presence of Ca^{2+} ion alone and V_{Mg} is that in the presence of both Ca^{2+} ion and Mg^{2+} ion, the inhibition curves form the first order sigmoid curves.

It was also stated in p. 32 that K_m values were not changed by Mg^{2+} ion, *i.e.*, Mg^{2+} ion had no effect on the binding of actomyosin with ATP. It may be taken, therefore, to indicate that the mechanism of the inhibition by Mg^{2+} ion is as follows:

$$M^* + Ca \underset{K_1}{\rightleftarrows} \overset{Ca}{M^*}, \qquad M^* + Mg \underset{K_2}{\rightleftarrows} \overset{Mg}{M^*}$$

$$M^*S + Ca \underset{K_1}{\rightleftarrows} \overset{Ca}{M^*S}, \quad M^*S + Mg \underset{K_2}{\rightleftarrows} \overset{Mg}{M^*}$$

$$M^* + S \xrightarrow[k_1^*]{} \overset{Ca}{M^*S} \xrightarrow[k_2^*]{} \overset{Ca}{M^*} + P$$

where K_1, K_2 are the dissociation constants.

The inhibition by Mg^{2+} ion does not suffer any alterations even when the K^+ concentration is changed (Fig 6) but the competition values of Ca^{2+} to Mg^{2+} are slightly changed with the pH variations (Fig. 7): that is, about $10^{1.6}$ at sufficiently acidic ranges of pH, about $10^{2.0}$ at sufficiently basic ranges of pH and an about mean value between them at pH 6.5.

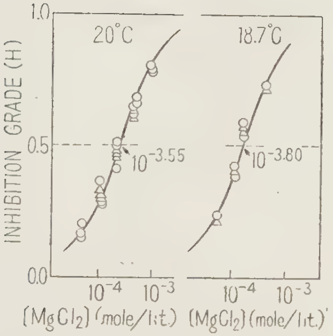

FIG. 6. Effect of the potassium concentration upon Mg-inhibition of ATPase. [K^+] 0.5 *mole/lit.* (○), 0.16 *mole/lit.* (△); veronal-acetate buffer at pH 6.5; [$CaCl_2$] 2×10^{-2} *mole/lit.* (left curve), 1×10^{-2} *mole/lit.* (right curve); [ATP] 1×10^{-3} *mole/lit.* [actomyosin] 0.445 mg. protein/ml.

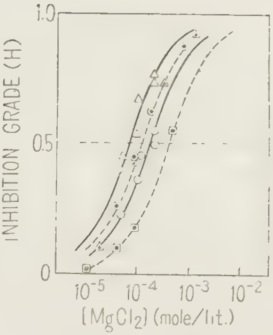

FIG. 7. Effect of pH upon Mg-inhibition of ATPase. Experiment I (full line) 19.7°, [$CaCl_2$] 1×10^{-2}*mole/lit.* [K^+] 0.48 *mole/lit.*, 0.22 mg. protein/ml., acetate buffer at pH 6.5 (○) and pH 9.1 (△). Experiment II (dotted line): 9.7°, [$CaCl_2$] 1.6×10^{-2} *mole/lit.*, [K^+] 0.16 *mole/lit.* ca. 0.3 mg. protein/ml. glycine buffer at pH 9.3 (⊙) and pH 8.0 (◬), veronal-acetate buffer at pH 5.5 (▣).

These results may be understandable simply by presuming that the ratio of the dissociation constant of Ca^{2+} to that of Mg^{2+} in M^*S^- is different from that in M^*S; that is, the ratio in M^*S^- is $10^{2.0}$ and $10^{1.6}$ in M^*S respectively.

It is further observed in Fig. 8 that the Mg^{2+} inhibition curves are shifted at the concentrations of enzyme protein over 0.22 mg per ml from such a fair Sigmoid curve that is obtainable below 0.22 mg. per ml. (♯).

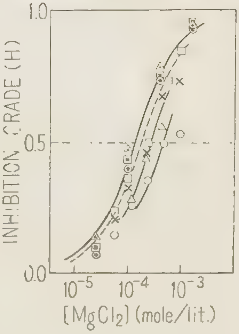

FIG. 8. Effect of enzyme concentration upon Mg-inhibition of ATPase. Experiment I; 8°, glycine buffer at pH 9.3, [K] 0.16 *mole/lit.*, ATP 4×10^{-4} *mole/lit.*, [$CaCl_2$] 3.2×10^{-2} *mole/lit.* [actomyosin] 0.4 mg. protein per ml. (⊙), 0.27 mg. protein/ml. (▣), 0.2 mg. protein/ml. (◬). Experiment II: [K] 0.48 *mole/lit.*, [$CaCl_2$] 1×10^{-2} *mole/lit.*, veronal-acetate buffer at pH 6.5, 15.5°, 0.22 mg. protein/ml. (□), at pH 6.1, 22.4°, 0.36 mg. protein/ml. (×), at pH 6.1, 21.3°, 0.72 mg. protein/ml. (△), at pH 6.5, 23°, 1.44 mg. protein/ml. (○).

♯ This is probably due to the reason reported by Koga & Maruo (*12*).

In order to compare the case of ATPase action with that of the light-scattering, the ratio of Mg^{2+} concentration to Ca^{2+} concentration is calculated to be about 1/10 from these results, under such conditions that pH 6.3, temperature at 23.5°, actomyosin 1.6 mg per ml, [KCl] $=0.48\ M$ and the ratio of Mg- to Ca-actomyosinate is 42.5 per cent.

Inhibition by Inorganic Pyrophosphate: The ATPase activity is inhibited by inorganic pyrophosphate (Pr) and the inhibition is that of second order reaction and also the effect of Pr is competitive with ATP as may be seen in Fig. 9a-b.

These results seem to suggest such a mechanism as follows:

$$M^* + S \xrightarrow[k_1^*]{} M^*S \xrightarrow[k_2^*]{} M^* + P$$

$$M^* + 2Pr \underset{K}{\rightleftarrows} M^*(Pr)_2$$

According to the above reaction schema, the inhibition grade H can be represented as:

$$H = \frac{[Pr]^2}{\phi + [Pr]^2}, \qquad \phi = K\left(1 + \frac{k_2^*}{k_1^*} \cdot [S]\right).$$

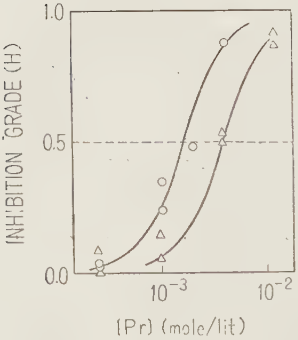

FIG. 9a. Inorganic pyrophosphate-inhibition of ATPase. 10° glycine huffer at pH 9.4, $[K^+]$ 0.18 *mole/lit.*, $[CaCl_2]$ 8.7×10^{-3} *mole/lit.*; [ATP] 2×10^{-4} *mole/lit.* (○) 4×10^{-4}*mole/lit.* (△).

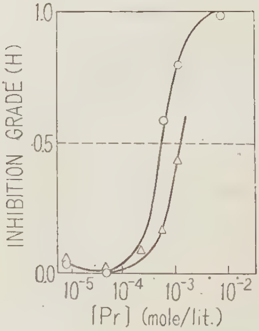

FIG. 9b. Inorganic pyrophosphate-inhibition of ATPase. 15°, veronal-acetate buffer at pH 6.5, $[K^+]$ 0.48 *mole/lit.*, 0.36 mg. protein/ml., $[CaCl_2]$ 2×10^{-2} *mole/lit.* [ATP] 2.5×10^{-4} *mole/lit.* (○), 1.3×10^{-3} *mole/lit.* (△).

This equation is in good agreement with the observed relation of H for [Pr] and [S]. It is found from our data by introducing $k_2^*/k_1^* = 1.5\times 10^{-4}$ *mole/lit.* to the above equation that $K=10^{-5.8}$ *mole/lit.*

THE CHANGE OF THE LIGHT-SCATTERING

Methods—The change of the intensity of the scattered light due to actomyosin solution upon the addition of ATP was traced with an electron multiplier-electromagnetic oscillograph (YEW, 3 elements, oscillator D-type) or μA-meter system. 0.5 ml. of ATP solution was breathed carefully into 14.5 ml. of the purified myosin B solution through a pipette whose tip was cut and the light-scattering of the protein solution as measured at an angle of 45° or 135° measured clockwise from the incident beam. Outline of the equipment for these measurements is shown in Fig. 9a. RCA-931A tube was used as an electron multiplier and its adjoining circuit is shown in Fig. 9b. The time for stirring is about 0.1 second and similar results were obtained at both angles, 45° and 135°. In so far as not mentioned specially all measurements were

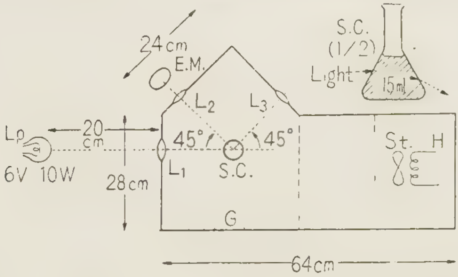
24cm
E.M.
S.C.
(1/2)
Light
15ml
L2
L3
Lp
20 cm
45°
45°
6V 10W
L1
S.C.
28cm
St.
H
G
64cm

FIG. 10a. Apparatus for measurement of the light-scattering. E.M.: electron multiplier, S.C.: sample vessel, Lp: lamp, L_1, L_2, L_3: lens, G: window, St.: stirrer, H: heater.

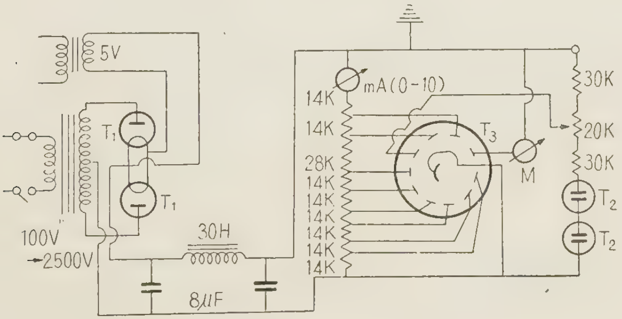
5V
T1
T1
100V
2500V
30H
8μF
14K
mA(0-10)
14K
28K
14K
14K
14K
14K
14K
14K
T3
M
30K
20K
30K
T2
T2

FIG. 10b. Adjoining circuit of an electron multiplier RCA-931-A. T_1: DC-762-A, T_2: VRB- 135/60, T_3: RCA-931-A, M: oscillograph or μA-meter.

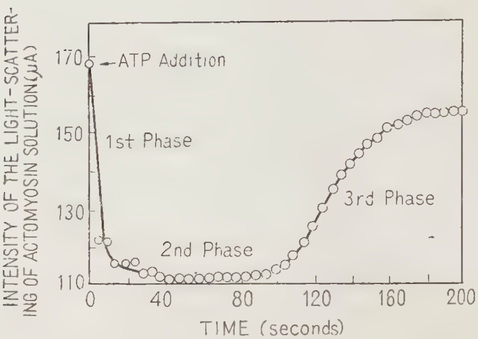

Fig. 11. Effect of ATP on the light scattering of actomyosin solution. 20°, pH 6.4, ATP 2.2×10^{-5} *mole/lit.*, [$MgCl_2$] $(2/3)\times10^{-2}$ *mole/lit.*, [KCl] 0.48 *mole/lit.*

carried out under the condition that temperature at 20°—21°, at pH 6.4 and [KCl]=0.4—0.5 *M*.

Results and Discussion—The time course of the light-scattering change, an example of which is shown in Fig. 11, can be divided into three phases, being similar to that of the viscosity change. When ATP is added to a solution of actomyosin, there is a rapid decrease in the light-scattering intensity (First phase); following this is a period during which the reduced intensity remains constant (Second phase); finally, when ATP added is split to a certain extent, the light-acattering intensity rises slowly (to about original level) (Third phase).

(a). *First Phase:* This change is completed within a few seconds or slightly over ten at most, while it takes about 20—30 seconds for every one of the viscosimetric measurements which have been used hitherto to investigate the deformation of actomyosin. Therefore, it has not yet been possible to measure the velocity of this change.

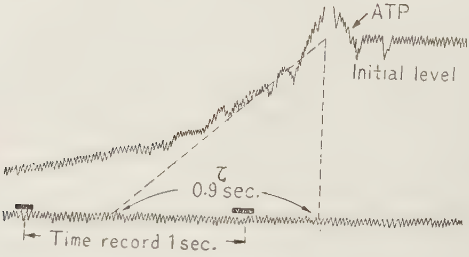

Fig. 12. An example of oscillogram of the light-scattering change of actomyosin solution. 20°, pH 6.4, [$MgCl_2$] 1/100 *mole/lit.*, [ATP] $(1/16)\times1.8\times10^{-4}$ *mole/lit.* Arrow on upper right indicates the time when ATP is added and base line corresponds to the intensity of the light-scattering after the addition of sufficient amount of ATP.

Fig. 12 shows an example of our results obtained by means of oscillography. Here, τ is the time taken, at initial velocity, to complete the light-scaterring change, *i.e.*, to reach the minimum value of the intensity observed upon the addition of ATP sufficiently in quantity. The relationship between the reciprocal of τ and the amount of ATP in presence of various cations is shows in Figs. 13a and 13b.

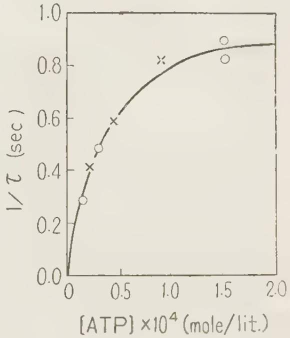

FIG. 13a. Relationship between initial velocity of the light-scattering drop (First Phase) and ATP concentration in the presence of K and Ca. 21°, pH 6.4, [K] 0.48 *mole/lit.* (○), [K] 0.48 *mole/lit.* plus [$CaCl_2$] 1/150 *mole/lit.* (×).

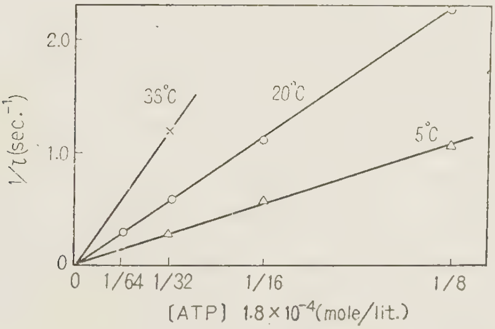

FIG. 13b. Relationship between initial velocity of First Phase of the light-scattering change and ATP concentration in the presence of 1×10^{-2} *mole/lit.* $MgCl_2$, pH 6.4, [KCl] 0.48 *mole/lit.*

When one takes into consideration that $1/\tau$ in the presence of K^+ ion and Ca^{2+} ion remains constant at higher ATP concentration and that the ATPase action occurs herewith, the following mechanism may be thought out:

$$M + S \xrightarrow[k_1]{} MS \qquad (1)$$

$$\mathrm{MS} \xrightarrow[k_2]{} \mathrm{M} + \mathrm{P} \quad (2)$$

$$\mathrm{MS} \xrightarrow[k_3]{} \mathrm{M^*S} \quad (2)$$

According to this reaction schema, the velocity of the light-scattering change (V) can be given by:

$$V = \frac{\mathrm{d\,[M^*S]}}{\mathrm{dt}} = k_3\,[\mathrm{MS}].$$

At the stationary state,

$$\frac{\mathrm{d\,[MS]}}{\mathrm{dt}} = k_1[\mathrm{M}][\mathrm{S}] - (k_1 + k_2) \quad [\mathrm{MS}] = 0$$

Hence,

$$V_{\mathrm{initial}} = \frac{k_3[\mathrm{M}]}{1 + \frac{k_2 + k_3}{k_1[\mathrm{S}]}}$$

$$\textit{i.e.,} \qquad \frac{1}{\tau} = \frac{k_3}{1 + \frac{k_2 + k_3}{k_1[\mathrm{S}]}}$$

From the facts described in the previous chapter, it is deduced that reaction (2) does not proceed in the presence of Mg^{2+} and that Mg^{2+} as well as Ca^{2+} does not affect reaction (1).

Based on the above deduction, the velocity constants can be estimated from our results; i.e.,

$${}_{\mathrm{K}}k_1 = {}_{\mathrm{Ca}}k_1 = {}_{\mathrm{Mg}}k_1 = 10 \times 10^4 (\textit{lit/mole·sec.})$$
$${}_{\mathrm{K}}k_2 = {}_{\mathrm{Ca}}k_2 = 7/3 \quad (\textit{sec.}^{-1}), \quad {}_{\mathrm{Mg}}k_2 \ll {}_{\mathrm{Mg}}k_3$$
$${}_{\mathrm{K}}k_3 = {}_{\mathrm{Ca}}k_3 = 1 \quad (\textit{sec.}^{-1}), \quad {}_{\mathrm{Mg}}k_3 \gg 5 \quad (\textit{sec.}^{-1})(\sharp)$$

Here, it is characteristic that Mg^{2+} ion accelerates reaction (3) remarkably. Ca^{2+} ion is competitive with Mg^{2+} ion. The values of τ upon the addition of 2×10^{-5} *mole/lit.* ATP at 23.5°, pH 6.3 are given in Table IV. Here, it can be interpreted that, when the ratio of the concentration of Mg^{2+} ion to that of Ca^{2+} ion added is 1 : 4, the ratio of Mg- to Ca-actomyosinate is 42.5 per cent.

Further, the activation energy of reactions (1) and (3) were estimated through the temperature dependence;

$${}_{\mathrm{Mg}}\Delta \mathrm{H}_1^* = 7.5 \text{ Kcal. } (\sharp\sharp)$$
$${}_{\mathrm{K}}\Delta \mathrm{H}_2^* \quad = 7.5 \text{ Kcal.}$$

♯ We symbolize the presence of K^+ ion or Mg^{2+} ion by the affix of K or Mg.

♯♯ ${}_{\mathrm{Mg}}\Delta \mathrm{H}_1^*$ represents the activation energy $\Delta \mathrm{H}^*$ for reaction (1) in the presence of Mg^{2+}.

TABLE IV

Effect of Ca^{++} and Mg^{++} on the Rate of the light-scattering change (First Phase)

(23.5° pH 6.3, [ATP] 2×10^{-3} *mole/lit.*, 1.6 mg. protein/ml.)

In the addition of	Time for the complete change with the initial velocity
$CaCl_2$ 1/400 *mole/lit.*	2.0 *second*
$MgCl_2$ 1/400 *mol/elit.*	0.42
$CaCl_2$ 1/100 *mole/lit.* $+MgCl_2$ 1/400 *mole/lit.*	0.77

(b). *Second Phase:* When ATP is present over a certain amount, a period appears during which the intensity of the light-scattering remains constant.

As pointed out by Csapó (*13*) in his viscosimetric work, the duration of this second phase is proportional to the amount of ATP added and is reversely propertional to the activity of ATPase; that is this phase corresponds with the reaction:

$$M^* + S \longrightarrow M^*S \longrightarrow M^* + P$$

(c). *Third Phase:* With the exception of the first few seconds in this phase when a small amount of ATP may still remain, the velocity of the recovery is independent of the initial concentration of ATP added and obeys the formula for a first order reaction and does not suffer much affect by the addition of Mg^{2+} or Ca^{2+}.

The velocity constant for the reaction:

$$M^* \longrightarrow M \quad (4)$$

can be estimated, that is:

$$_{K}k_4 = 1/40 \ (sec.^{-1})$$
$$_{Ca}k_4 = 1/30 \ (sec.^{-1})$$
$$_{Mg}k_4 = 1/30 \ (sec.^{-1})$$

The activation energy for this reaction is found to be:

$$_{Mg}\Delta H_4^* = 4.1 \text{ Kcal.}$$

The Change of Light-scattering upon the Addition of Inorganic Pyrophosphate: In the case of addition of ATP, the true equilibrium of the combination between ATP and actomyosin is not ascertainable because ATP is split. Inorganic pyrophosphate (Pr), on the other hand, is not split by actomyosin but gives rise to the light-scattering change of actomyosin solution only in the presence of Mg^{2+} ion.

Moreover, the deformation of actomyosin particles upon Pr-addition seems to be the same as upon the addition of ATP; *i.e.*, as in Table V, the degree of decrease in the light-scattering intensity upon the addition of the sufficient amount of Pr is the same as that of ATP.

TABLE V

The Light-scattering Change upon the Addition of ATP and Inorganic Pyrophosphate (Pr)

Angle ⟶	45°	135°
Pr	38	37
ATP	36	35

The numerical value $\frac{A-B}{A}\times 100$. A: Initial intensity of the light-scattering of actomyosin solution before the addition of ATP or Pr. B: Minimum intensity of the light-scattering of actomyosin solution after the addition of ATP or Pr.

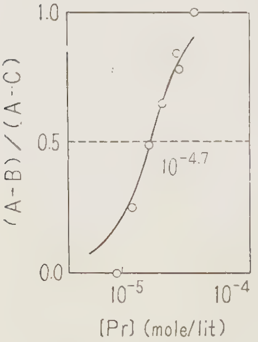

FIG. 14a. Effect of inorganic pyrophosphate (Pr) on the light-scattering of actomyosin solution (0.38 mg. pretein/ml.) in the presence of 1×10^{-2} *mole/lit.* $MgCl_2$. 12°, pH 6.4, [KCl] 0.48 *mole/lit.* A: Initial intensity of the light-scattering of actomyosin solution before the addition of ATP or Pr. B: The light-scattering intensity of actomyosin solution after Pr addition. C: The light-scattering intensity of actomyosin solution after the addition of the sufficient amount of ATP.

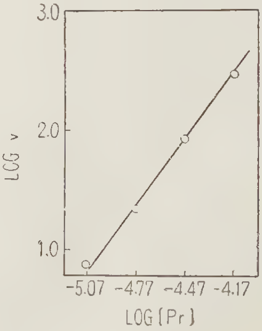

FIG. 14b. Relationship between initial velocity of the light-scattering drop and concentration of inorganic pyrophosphate (Pr) in the presence of 1×10^{-2} *mole/lit.* $MgCl_2$.

As may be seen in Fig. 14a, the relationship between the percentage of the light-scattering intensity after Pr-addition to the original intensity of the actomyosin solution and the concentration of Pr added is shown as a second order sigmoid curve.

Thus, the dissociation constant for the reaction:

$$M + 2Pr \rightleftarrows M^* (Pr)_2$$

is found to be $10^{-9.4}$ *mole/lit.*

Moreover, the initial velocity of deformation of actomyosin particles upon Pr-addition is proportinal to $[Pr]^2$ as may be seen in Fig. 14b.

THE SORT AND THE NUMBER OF ACTIVE CENTERS

Sort of Active Centers—How is the deformation of actomyosin particles related to the ATPase action ? The question of whether ATP combines with the same action point (active center) of actomyosin molecule in both phenomena or with the different points of action is very important in order to ascertain the mechanism of these reactions.

In the mechanism described in the former chapter, it has been assumed tacitly that these two active centers are identical with each other. But even if we take the two active centers to be different, it may be possible to explain the experimental results although a more complicated mechanism must be thought out in this case.

However, the following results observed in both phenomena favour the view that the two active centers are identical:

(i) The reaction velocity is of the first order with respect to ATP concentration when [ATP] is low..

(ii) Mg^{2+} ion and Ca^{2+} ion are bound by actomyosin competitively with each other and noncompetitively with ATP and their binding is of the first order.

(iii) Pyrophosphate is bound competitively with ATP and its binding is of the second order.

As in Table VI, the velocity constants and the dissociation constants have the similar features in both cases and here, the slight variations of numerical values may come from the situation that the original actomyosin M takes the leading part in the light-scattering change, while the deformed actomyosin M* takes the leading part in the ATPase action.

Number of Active Centers per One Molecule of Myosin—The number of active centers per one molecule of myosin was estimated by means of the following procedure.

TABLE VI

Reaction	k or K	Reaction	k or K
$M+S\rightarrow MS$	${}_{\mathrm{K}}k={}_{\mathrm{Ca}}k={}_{\mathrm{Mg}}k$ $=10\times10^4$ (*lit./mole sec.*)	$M^*+R\rightarrow M^*S$	${}_{\mathrm{K}}k={}_{\mathrm{Ca}}k={}_{\mathrm{Mg}}k$ $=5\times10^4$ (*lit./mole sec.*)
$MS\rightarrow M+P$	${}_{\mathrm{Ca}}k={}_{\mathrm{K}}k=7/3$ (1/*sec.*)	$M^*S\rightarrow M^*+P$	${}_{\mathrm{Ca}}k=7$ (1/*sec.*)
$MS\rightarrow M^*S$	${}_{\mathrm{Ca}}k={}_{\mathrm{K}}k=1$ ${}_{\mathrm{Mg}}k\gg5$ (1/*sec.*)	$M^*\rightarrow M$	${}_{\mathrm{K}}k=1/40$ ${}_{\mathrm{Ca}}k={}_{\mathrm{Mg}}k=1/30$ (1/*sec.*)
$\overset{\mathrm{Ca}}{M}+Mg^{++}\rightleftarrows\overset{\mathrm{Mg}}{M}+Ca^{++}$	$K\fallingdotseq1/4$	$\overset{\mathrm{Ca}}{M^*}+Mg^{++}\rightleftarrows\overset{\mathrm{Mg}}{M^*}+Ca^{++}$	$K\fallingdotseq1/10$
$M+2Pr\rightleftarrows M^*Pr_2$	$K=10^{-9.4}$ (*mole/lit.*)^2	$M^*+2Pr\rightleftarrows M^*Pr_2$	$K=10^{-5.9}$ (*mole/lit.*)^2

Plotting the quantity of ATP added in the presence of Mg^{2+} ion on the abscissa and the ratio of the minimum intensity to the original intensity of the light-scattering on the ordinate gives a broken line as shown in Fig. 15a.

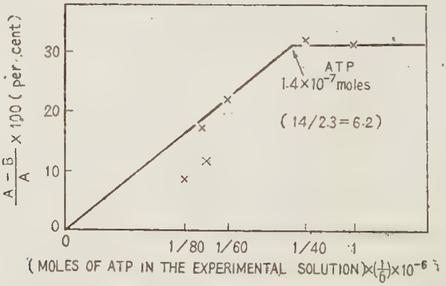

FIG. 15a. Actomyosin- ATP combination in the presence of 1/400 *mole/lit.* $MgCl_2$. 21°, pH 6.4, KCl 0.48 *mole/lit.* moles of myosin in the experimental solution$=\dfrac{25.9\times10^{-3}\times(3/4)}{8.4\times10^5}=2.3\times10^{-8}$. A: initial light-scattering intensity of actomyosin solution before ATP addition. B: the light-scattering intensity of actomyosin solution of phase II.

(♯) This lower drop is probably due to such a situation that the ATPase activity can not be neglected at the lower concentration of ATP.

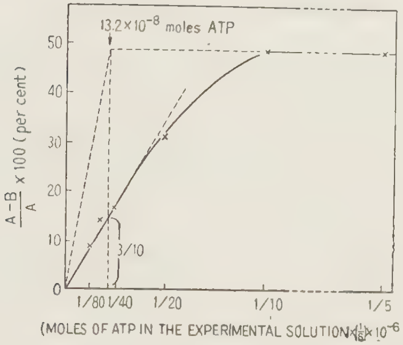

FIG. 15b. Actomyosin-ATP combination in the presence of Mg (dotted line) and in the absence of Mg (×). 21°, pH 6.4, [KCl] 0.48 *mole/lit.* Moles of myosin in the experimental solution $\frac{25\times 10^{-3}\times (3/4)}{8.3\times 10^{5}}$ $=2.2\times 10^{-6}$. A: Initial light-scattering intensity of actomyosin solution before ATP addition. B: Minimum light-scattering intensity of acmyosin solution after ATP addition.

Since ATPase activity in the presence of Mg^{2+} ion is negligible as proviously described, the above results may be interpreted as indicating that the reaction $M+S\rightarrow M^*S$ proceeds in proportion to the concentration of ATP.(♯) Thus, the quantity of ATP at the breaking point should be equal to the quantity required for the complete deformation of all the myosin present to M*S.

Based on the above results, presuming that molecular weight of myosin is 840,000 (*14*) and that the myosin to actin ratio in myosin B used in our experiments 1:3, the number of ATP molecules required for the complete deformation of one molecule of myosin is calculated to be:

$$\frac{1.4\times 10^{-7}}{2.3\times 10^{-8}}=6.2\fallingdotseq 6,$$

that is, it comes to this, that the number of active centers, in other words, the number of units per one molecule of myosin, is six.

On the other hand, it is well known (*15*) that actomyosin is a stoichiometric complex which contains myosin (M) and actin (A) at the ratio of 2.5: 1 and that molecular weight of actin is about 70,000 (*16*); that is, it comes to this, that six molecules of actin combine with one

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♯ From this fact, the reverse reaction of the reaction (1*) is neglected by us. See p. 31.

molecule of myosin, in other words, one molecule of actin with one unit of myosin.

Such being the case, we shall use the expression " AM " for the unit of actomyosin from now on.

Mommaerts (*17*), who carried out similar work using the viscosimeter, reported that one ATP combined with every 300,000 g. myosin. But his figure is not acceptable from the following view-points:

(i) In the ATP concentration in question, the viscosity drops to minimum level within several seconds after the ATP addition and then rises immediately. Therefore, the direct measurement of the minimum value by means of viscosimetry is impossible.

(ii) At 0°, at which his work was done, most of the actomyosin particles dissociate to actin and myosin (even in the absence of ATP) as will be mentioned later.

Then, Fig. 15b shows that upon the addition of ATP in quantity just to induce the complete deformation of actomyosin in the presence of Mg^{2+}, only three-tenths of the actomyosin deforms in the absence of Mg^{2+} ion. Considering the fact as described formerly that under these conditions, the two reactions:

$$\begin{tikzcd} & M + P & & (2) \\ MS \arrow[ur] \arrow[dr] & & & \\ & M^*S \arrow[r] & M^* + P & (3) \end{tikzcd}$$

occurred coincidently and the ratio of the velocity constant of the reaction (2) to reaction (3) was 7:3, this observation described above may be easily understandable.

MECHANISM OF MUSCULAR CONTRACTION

Based on the mechanims of ATP-actomyosin interactions as discussed in former chapters, we will discuss the mechanism of muscular contraction in this chapter.

What physical meaning has the deformation of actomyosin;

$$M \longrightarrow M^*$$

which is induced by the addition of ATP? Experimental answers to this question have already been sought by various methods; viscosimetry (*18*), ultra-centrifugation (*18*) and electron microscopy (*19*).

Generally speaking it has been hither-to emphasized that ATP addition causes a disaggregation of actomyosin into actin and myosin. But the enzymatic property of the myosin produced by this assumed disaggregation is different from that of the single myosin because it has been reported that the behaviour of acotmyosin-ATPase towards Mg^{2+} ion and pH is different from that of myosin-ATPase (*20*). J.J. Blum (personal communication) states that the ATP addition causes a reversible shape change at constant molecular weight, indicating no depolymerization into " actin " and " myosin A." Therefore, the expressin $AM \longrightarrow AM^*$ instead of $M \rightarrow M^*$ will be used hereafter.

It is further well known that in $[KCl]+[NaCl] \fallingdotseq 0.15\ M$ this being the concentration observed in the muscle (*21*), ATP addition causes so-called " Superprecipitation " of actomyosin. It is also evident in the electron microscopic study by Astbury *et al.* (*19*) that this phenomenon involves at least two steps; once a deformation of actomyosin and then a new polymerization.

Myosin and actin in resting muscle are associated in the state of actomyosin (*22*). Muscle contains a considerable quantity of Mg^{2+} and Ca^{2+}, most of which are bound by actomyosin and other protein in muscle (*23*). Thus, the concentration of free ions must be very low and therefore even a small change of the binding force between these ions and actomyosin may have affect on the concentration of free ions.

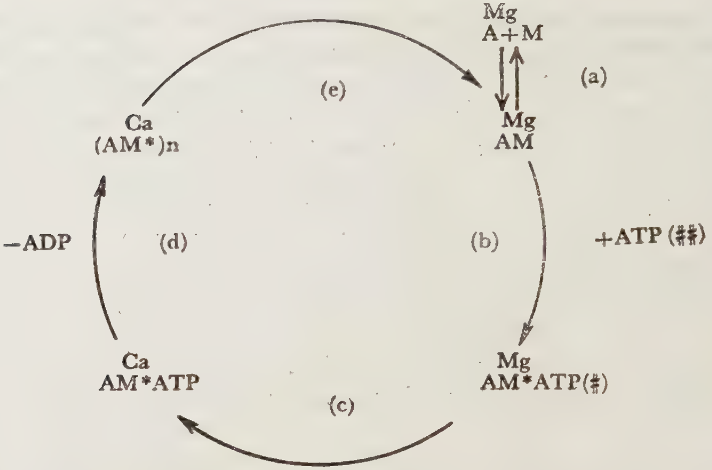
On the assumption that the ratio of the binding force of Mg^{2+} to Ca^{2+} by actomyosin is much larger in rest than in contraction, it may be possible that the ratio of free Mg^{2+} ion to free Ca^{2+} is more than 1/4 in rest and less than 1/10 in contraction. In such a case, actomyosin will be present as Ca-actomyosinate in contraction and as Mg-actomyosinate in rest. The facts that there are both the increase of free Ca^{2+} (*24*) and the decrease of free Mg^{2+} (25) with muscular activity may favour the above assumption.

Putting together the whole story of the above discussions, the interaction of actomyosin to ATP under such a condition as in muscle may be expressed as follows:

Sequence of Events in Muscular Contraction—It has been known for a long time that upon stimulating muscle there is a latent period for about 3—5 milliseconds and then contraction and finally relaxation. It takes about 1/10 seconds for one twitch.

Now, let us assume that when stimulated, a part of ATP bound by actomyosin and by the other proteins is set free and then follow such reactions as described in the former parts of this chapter. Thus, if the reaction (d) corresponds to contraction, then the reactions (b) and (c) are involved in latent period and the reaction (e) in relaxation.

The uniform concentration of ATP set free on that occasion may



be, of course, far less than the total ATP concentration as observed in striated muscle, that is, 5×10^{-3} *mole/lit.* However, the activity of ATP involved in the reaction (b) must be far more than that uniform concentration because the distribution of ATP is not uniform and is high in actomyosin surroundings. Therefore, it may be estimated to be about 5×10^{-3} *mole/lit.*

If this is the case, the velocity of the reaction (b) comes to about $10\times10^{4}\times5\times10^{-3}=5\times10^{2}$ *sec.*$^{-1}$ which is in good harmony with the fact that the latent period (the interval between stimulus and onset of contraction) is about 3—5 m sec. An already-known fact that on increasing temperature by 10°, there was a reduction by about 2/3 of the latent period (27) is also in accord with our already-described result that ΔH^* of the reaction (b) was 7.5 Kcal.

Further, the facts that the duration of shortening is 0.05 sec. and is reduced to a half by 10° increase are well corespondent to such results that the velocity constant is 42 *sec.*$^{-1}$ (glycine buffer, 0.16 KCl, 37°) and ΔH^* is about 12 Kcal in the ATPase action.

# The existence of the complex AM*ATP is incompatible with the theory of Bailey and Perry (*26*) according to which it is one and the same SH-group which is responsible for splitting ATP and linking actin to myosin, the two functions competitive with one another.

## In addition of one ATP per one unit of actomyosin, many ATP adsorbed on one actomysin are required for the contraction but they are unchanged in this contraction cycle. Therefore, they are left neglected in this paper.

It may be due to the difference between the interrelation of actomyosin particles to each other in the extracted actomyosin system and that in intact muscle that the relaxation in the extracted actomyosin system is very slow. The situation that relaxation depends delicately upon the structure of actomyosin in muscle is also made clear through the fact that even intact fibrers when made to shorten to an excess degree, do not relax any more (*28*).

Recently, A. V. Hill (*29*) reinvestigated the early heat production during an isotonic twitch. His results reveal the nature of the first event in heat production, the heat of activation. Such heat appears during the mechanical lag period and starts off at a maximum rate after a very short latency. It seems to be quite independent of the conditions of muscle contraction. Its value is 3 millicalories/g. muscle, that is 5.7 Kcal./140,000 g. muscle myosin per one twitch.

The heat produced during a twitch comprises the heat of activation and the heat of shortening. The latter is proportional to the shortening and primarily independent of the work alone. No heat is produced during relaxation if the load lifted by the muscle is detached before relaxation begins. If the load is left on, the equivalent of its potential energy is released during relaxation, but no more. These observations can be interpreted by the following postulate; heat of activation is attributed to heat content ΔH of the exothermic reaction (b) and heat of shortening to the exothermic reaction (d) whereas the fact that during relaxation no heat is produced is harmony with the results that ΔH^* for the reaction $M^* \longrightarrow M$ is very small.

D. K. Hill (*30*) stated that in a single twitch the contraction is accompanied by an increased transparency, roughly coincident with the latency relaxation, followed by an increased turbidity when contraction occurs. This, then, has to change back again in the opposite sence. These observations are interpreted by the previously mentioned correspondence of the changes of actomyosin to the phases of muscular contraction, that is, the light-scattering intensity decreases by the reaction (b), increases by the reaction (d) and changes back again by the reaction (e).

On the other hand, the change of the light-transparency of muscle in tetanus (*31*) may rise in a different mechanism from that in twitch.

ATP Consumption—

ATP Amount Involved in A Single Twitch: According to Lundsgaad (*32*), a striated muscle in iodoacetate poisoning performs 50 full twitches anaerobically. Then, the contents of ATP and phosphocreatine (CP) known to be 5×10^{-6} and 20×10^{-6} *moles per g.* of muscle, respectively.

The followed three reactions are considered as those possible to occur in such an iodoacetate poisoning muscle;

(i) $\mathrm{ATP} \longrightarrow \mathrm{ADP} + \mathrm{P}$ (Adenosine triphosphatase)
(ii) $\mathrm{CP} + \mathrm{ADP} \longrightarrow \mathrm{C} + \mathrm{ATP}$ (Creatine-ATP-phosphorase)
(iii) $2\mathrm{ADP} \longrightarrow \mathrm{ATP} + \mathrm{AMP}$ (Myokinase)

When the twitches are repeated, the reactions (i) and (ii) proceed completely and the reaction (iii) proceeds only incompletely.

Thus, it comes to this, that the quantity of ATP split for one twitch is more than $5\times10^{-7}=(25\times10^{-6})/50$ moles per g. of muscle, that is $0.9=(5\times10^{-7}\times140{,}000)/0.075$ (#) moles per 140,000 g. of myosin and less than 1.8 moles per 140,000 g. of myosin.

Csapó (*33*) conducted a similar experiment with uterus and reported that uterine muscle in monoidoacetate poisoning performs seven full twitches (ten twitches) anaerobically and that the content of CP plus ATP in uterine muscle is 2.6×10^{-6} moles per g. of muscle, This result indicates the consumption of $0.7=(2.6\times10^{-6}\times140{,}000)/(7\times0.085)$—1.4 moles ATP per 140,000 g. of myosin for one full twitch.

Recently, Mommaerts (*34*) estimated the distribution of adenine nucleotides of frog muscle, which was fixed at the hight of contraction by means of putting the muscle in liquid air, and found that about one-fifth of the ATP content in the muscle, that is about $10^{-6.0}$ mole ATP per g. of muscle$=1.8$ moles ATP per 140,000 g. of myosin were split to ADP (AMP production is negligible during this one twitch).

On the other hand according to our theory, the minimum quantity of ATP involved in a single full twitch is one mole per 140,000 g. of myosin. It is, therefore, deduced that the ATP consumtion during muscular contraction in vivo is very economical and about 1.5 moles ATP per unit of myosin (though these figures vary a little (1—1.8 moles) according to the condition under which the twitch is induced) are consumed for one twitch(##).

Efficiency of Contracting Muscle: Varga (*35*) measured the reversible work of the glycerol-extracted musclus psoas of the rabbit and found that

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[#] It is assumed that the whole muscle contains 20 per cent of proteins composed of 50 per cent myosin B in which the ratio of action to myosin is 1:3.

[##] This fact that ATP in amounts over one mole per unit myosin is consumed during one twitch in vivo may be due to the proceeding of the reaction ($\mathrm{AMS}\rightarrow\mathrm{AM}+\mathrm{P}$) to a few extensions or to the repeating of the reaction ($\mathrm{M}^*+\mathrm{S}\longrightarrow\mathrm{M}^*\mathrm{S}\longrightarrow\mathrm{M}^*+\mathrm{P}$).

it is about 9 Kcal per unit of myosin in the complete contraction(#). Similar values are also observed with rat muscle and frog muscle, that is about 9.0 Kcal with rat diaphragms and about 9.3 Kcal. with frog sartorious.

On the other hand, ΔF change for the ATP splitting is about 12 Kcal per mole. Therefore, the efficiency of muscular contraction comes to be $9/(12\times1.5)=50$ per cent; indicating a good agreement with the experimental results (*38*).

Breakdown of ATP in Active Muscle: For mammalian muscle at 37°, the rate of the breakdown of ATP in active muscle was estimated to be a value of the order of 10^{-3} mole per minute per gram of muscle (*18*). On the other hand, the velocity constant of ATP splitting per unit myosin by Ca-actomyosinate (##) was estimated to be about 42 $sec.^{-1}$ in the presence of glycine, at 37°, at pH 7.0. Further the number of the units of myosin per gram of muscle is estimated to be $(0.075\times6)/(8.4\times10^5)$. Therefore, the rate of the breakdown of ATP by Ca-actomyosinate comes to be $42\times60\times(0.075\times6)/(8.4\times10^5)=1.4\times10^{-3}$ moles per minute per g. of muscle; that is, it agrees approximately with the above results.

Temperatur Effect of Muscular Contraction—Laki *et al.* (*39*) reported at 26.5°, the larger part of myosin was combined with actin and at 4.9°, only a small amount of actomyosin was formed; that is, ΔH of the reaction (a) $A+M\rightleftarrows AM$ is very large (endothermic).

Therefore, on the assumption that when ATP is added to actomyosin, AM contracts and M does not, the contractibility of actomyosin thread and muscle upon the addition of ATP should be strikingly changeable with temporature-variations. In fact, this is established by Szent-Györgyi's scholars (*45*). They have regarded the temperature dependence of the contractibility as indicative of that of the equi librium reaction; AM relaxation$\rightleftarrows$AM contraction, but it should be, of course, interpreted as indicating that of the reaction (a).

Further, they concluded, through the comparison of the temperature dependence of the contractibility with that of the reversible work, that one mole of myosin is composed of 12 units on the assumption of 40

# He seems to postulate according to Weber (*36*) that the total muscle protein contains about 40 per cent myosin B. Since this content is evidently too small (*37*) we re-calculated the work from Varga's results on the assumption that the content of myosin B is 50 per cent. The value of work per unit of myosin is only approximate because the measurements of the shape and the length of muscle used are considerably uncertain.

## As mentioned previously, actomyosin is combined with Ca^{2+} in contraction.

per cent myosin B content in the total muscle protein, but it is also possible to explain their results even if it is assumed that 50% of the total muscle protein consists of myosin B and that one mole of myosin is composed of 6 units.

SUMMARY

With respect to the ATP splitting and the light-scattering change upon the addition of ATP to actomyosin solution, the relationship between the velocity of these two phenomena and the ATP concentration, in company with the function of inorganic pyrophosphate and some cations, were analysed kinetically and then the mechanism of the two phenomena was proposed.

It is also suggested that the ATP attacking points of actomyosin in the two phenomena are identical with each other and that the number of the active centers per one myosin molecule is six.

The mechanism proposed is able to illustrate the various facts observed about muscular contraction.

Finally, we are much indebted to Dr. Juro Horiuchi, the Head of the Research Institute for Catalysis, Prof. Hiroshi Tamiya, of Botanical Department, Faculty of Science (Tokyo University) and Prof. Naomoto Takasugi, of Chemical Department, Faculty of Science, for the facilities they have provided for this work and to Messrs Kinjiro Sukegawa and Junshiro Makino for valuable technical assistance.

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ADDENDUM

After this paper was written, we read Bozler's very interesting article "*Evidence for an ATP-Actomyosin Complex in Relaxed Muscle and Its Response to Calcium Ions*" (*Am J. Physiol.*, 168 760, 1952).

In his studies with the glyceroled muscle fibers, it was found 1) that in the relaxed condition the contractile elements are present in an activated state, possibly brought about by chemical combination of ATP with the contractile proteins. 2) that magnesium ions are essential for maintaining this state, and 3) that calcium ions in very low concentrations cause rapid contraction of this activated system, even in the absence of free ATP.

According to our mechanism (p. 47), when $k_d \ll k_e$ the muscle is in the state of relaxation and when $k_d \gg k_e$ it is present in the state of contraction.

As mentioned previously, the reaction (d) is inhibited by Mg^{2+} and is activated by Ca^{2+}. On the other hand, the reaction (e) is scarecely affected by these ions. Therefore, Bozler's results are illustrated by the following postulates: ${}_{Mg}k_d \ll {}_{Mg}k_e = {}_{Ca}k_e \ll {}_{Ca}k_d$.

The detailed discussion about the effects of divalent ions on glycerol-extracted muscle and Myosin B thread will be described in the next paper.

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MOLAR RATIO OF INDIVIDUAL BASES IN YEAST RIBONUCLEIC ACID

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Recently, a number of investigators have attempted to analyze quantitatively the purine and pyrimidine bases in nucleic acids. The results of Chargaff's work (*1*) involving the application of paper chromatography and Abrams' work (*2*) employing the isotope dilution method are, in general, not compatible with the tetranucleotide hypothesis in which the individual bases occur in 1:1:1:1 ratios. Gulland (*3*) has already pointed out that the tetranucleotide hypothesis is incompatible with the analytical data of nucleic acids. But the analytical data of yeast ribonucleic acid, especially that of Merck, are said to be accordant with the tetranucleotide structure in acidity by being four basic, in four acidic radicals (*4*) found afresh on its hydrolysis, in diffusion constant (*5*) determined by Lamm-Polsen's method, and in active hydrogens (*6*) determined by deuterium oxide, despite of the absence of adequate analytical data of individual bases. Therefore we tried to decide the molar ratio of individual bases of yeast ribonucleic acid from the analytical value of nitrogen, phosphorus, aminonitrogen, purine-nitrogen and guanine. The result suggests that adenine, guanine, cytosine and uracil in yeast ribonucleic acid (Merck) occur in nearly equimolar ratio.

EXPERIMENTAL

Material—Yeast ribonucleic acid prepared by Bauman's method from yeast and that of Merck were used. They were purified by Makino's method (*7*).

Merck	N, 12.86 %	
	P, 7.56 %	N:P=1.70:1
The other	N, 14.76 %	
	P, 8.7 %	N:P=1.68:1

Determination of Guanine—Hypo-iodite method of Willstätter and Schudel (*8*) has been used favourably for the determination of aldose and in 1932 its modification was applied to the study of purines by Grynberg (*9*). He postulated that guanine and xanthine were susceptible to this oxidation reaction employing iodine in weak alkaline solutions, and that 4 atoms of iodine were consumed per 1 mole of the substrate, but adenine and all the nucleosides and nucleotides, (even guanine nucleosides and guanine nucleotides,) did not undergo oxidation (Table I). We confirmed this finding experimentally and applied it to the determination of guanine in hydrolyzate of yeast ribonucleic acid.

TABLE I

Attitude of Purine and its Derivatives toward Hypoiodite

Experiment No.	Sample	Weight	$N/10$ Iodine consumed		Error	Iodine consumed per 1 mole of substrate
			Theoretical value	Experimental value		
		mg.	*ml.*	*ml.*	*per cent*	
1	Guanine-HCl	5.0	1.06	1.06	0	4 I
2	"	5.0	1.06	1.04	−1.9	4 I
3	Guanine	10.1	2.63	2.66	+1.0	4 I
4	Adenine	9.6	0	0.06	+1.7	0
5	"	11.30	0	0.016	+0.9	0
6	Xanthine	10.0	2.52	2.48	−1.5	4 I
7	"	10.0	2.50	2.48	−1.5	4 I
8	Hypoxanthine	12.6	0	0.03	+1.8	0
9	"	7.8	0	0	0	0
10	Adenosine	5.0	0	0	0	0
11	"	5.0	0	0	0	0
12	Guanosine	11.40	0	0	0	0
13	"	11.80	0	0	0	0
14	Guanylic-acid	13.00	0	0	0	0
15	Adenylic-acid	5.00	0	0	0	0

Procedure: Hydrolysis of yeast ribonucleic acid, introducing of the hydrolyzate to a centrifuge tube and neutralization are carried out through the procedure to be described in the following section of "determination of purine nitrogen." Next, the hydrolyzate is mixed with the precipitation reagent which consists of equal volume of ammoniacal silver nitrate solution and magnesia mixture, usually, 5–6 ml. of the precipitation reagent per 20 mg. of purines, and the resulting mixture is left to stand for about two hours with a cover. The precipitated silver purines are collected and washed three times with 3–5 ml. of the precipitation reagent using on centrifuge. The washed precipitate is suspended in 10 ml. of distilled water and to it 0.5 ml. of 25 per cent hydrochloric acid is added. Then the mixture is heated in the boiling water for one minute and is left to stand for one hour at room temperature. The silver chloride precipitated is centrifuged and the supernatant is introduced quantitatively into a Kjeldahlflask through paper filter and the silver chloride is washed three times with *N*/10 hydrochloric acid. The supernatant and the washing should contain guanine, adenine *etc*. One ml. of about 33 per cent sodium hydroxide is added to expell ammonia by making it alkaline under a reduced pressure at 40°. The substrate, insoluble in the warm alkaline solution (60–70°), is removed by filtration. When the supernatant is neutralized with 5 per cent sulfuric acid and left to stand at room temperature, the crystal of purine appears floating. A small amount of 4 per cent sodium hydroxide solution is added to it until the crystal of purine disappears. Next, *N*/10 iodine solution (usually 2.0—4.0 ml.) is added and the resulting solution is left to stand for 20 minutes at room temperature. After acidifying it with 5 per cent sulfuric acid, the iodine separated is titrated by *N*/10 or *N*/20 sodium thiosulfate using 2 per cent starch solution as an indicator and the guanine content is calculated from the data of iodine consumption. (Tables II, III, IV.)

TABLE II

Determination of Guanine

Experiment No.	Sample weight	Hydrolysis			*N*/10 Iodine consumed	Guanine	Error
		10% Sulfuric acid	Temperature	Time			
	mg.	*ml.*	°*C*	*minutes*	*ml.*	*mg.*	*per cent*
1	Guanine 21.65	2.2	100	120	5.646	21.47	−0.83
2	Adenosine 12.42	1.24	100	45	0.01	—	—
3	Guanosine 11.80	1.2	100	45	1.398	5.32	−4.8
4	Guanosine 19.85 Adenosine 18.25	4.0	100	45	2.38	9.04	−3.4

TABLE III

Guanine of Yeast Ribonucleic Acid Prepared by Bauman's Method and Purified according to Makino's Method

Experiment No.	Weight	Hydrolysis			$N/10$ Iodine consumed	Guanine	Guanine per 100 mg. of RNA.
		10% Sulfuric acid	Temperature	Time			
	mg.	*ml.*	°C	*minutes*	*ml.*	*mg.*	*mg.*
1	20.00	2.00	100	15	0.533	2.03	10.15
2	18.70	1.87	100	30	0.507	1.93	10.32
3	13.01	1.30	100	45	0.358	1.36	10.45
						Average	10.31

TABLE IV

Guanine of Yeast Ribonucleic Acid of Merck Purified according to Makino's Method

Experiment No.	Weight	Hydrolysis			$N/10$ Iodine consumed	Guanine	Guanine per 100 mg. of RNA.
		10% Sulfuric acid	Temperature	Time			
	mg.	*ml.*	°C	*minutes*	*ml.*	*mg.*	*mg.*
1	21.15	2.12	100	15	0.500	1.901	8.99
2	23.41	2.34	100	30	0.540	2.053	8.77
3	14.10	1.41	100	45	0.343	1.304	9.25
						Average	9.00

Determination of Purine Nitrogen—Purines of yeast ribonucleic acid were precipitated and determined by the copper-bisulfite method of Hitchings and Fiske (*10*). Yeast ribonucleic acid was hydrolyzed by 10 per cent sulfuric acid (*11*) for 15–60 minutes, favourably 45 minutes in boiling water. This time of hydrolysis was decided experimentally by the analytical value of purine nitrogen.

Procedure: Yeast ribonucleic acid is hydrolyzed with 10 per cent sulfuric acid, usually, 1 ml. per 10 mg. of the sample, for 15–60 minutes, favourably 45 minutes in a tiny sealed test-tube immersed in vigorously boiling water. Then the hydrolyzate in the test-tube is completely introduced into a 50 ml. centrifuge tube (with conical tip) with a few ml. of 4 per cent sodium hydroxide solution and 20–30 ml. of distilled water. It is then neutralized by using phenolphthalein as an indicator. The contents of the tube is next heated in a boiling water bath, and the purine bases are precipitated by adding 0.8 ml. of saturated solution of sodium bisulfite and 1 ml. of 10 per cent $CuSO_4 \cdot 5H_2O$. After 3 minutes heating, the precipitate is collected by centrifuge and washed twice with 10 ml. of hot water. This final precipitation is analyzed for nitrogen content by the Kjeldahl method (Tables V, VI, VII).

TABLE V

Purine Nitrogen

Experiment No.	Sample weight	Nitrogen contained (theor. value)	Hydrolysis			$N/10\ H_2SO_4$ employed (Kjeldahl)	Purine nitrogen	Error
			10% Sulfuric acid	Time	Temperature			
	mg.	*mg.*	*ml.*	*minutes*	*°C*	*ml.*	*mg.*	*per cent*
1	Guanine 4.12	1.91	0.41	60	100	1.316	1.84	−3.7
2	Adenine 11.54 Guanine 8.65	$\left.\begin{array}{l}4.27\\+\\4.01\end{array}\right\}8.28$	dissolved in 5.0 ml. *N*/10 NaOH			5.792	8.11	−2.1
3	Guanosine 15.00 Adenosine 16.49	$\left.\begin{array}{l}3.29\\+\\4.05\end{array}\right\}7.34$	5.0	45	100	5.104	7.15	−2.6
4	Guanosine 7.60 Adenosine 9.40	$\left.\begin{array}{l}1.67\\+\\2.31\end{array}\right\}3.98$	4.0	45	100	2.764	3.87	−2.8

TABLE VI

Purine Nitrogen of Yeast Ribonucleic Acid Prepared by Bauman's Method

Experiment No.	Yeast ribonucleic acid	Hydrolysis			$N/10\ N_2SO_4$ employed (Kjeldahl)	Purine nitrogen	Purine nitrogen per 100 mg. of RNA.
		10% Sulfuric acid	Time	Temperature			
	mg.	*ml.*	*minutes*	*°C*	*ml.*	*mg.*	*mg.*
1	13.50	1.35	4	100	1.893	1.061	7.86
2	10.05	1.00	15	100	1.787	1.001	9.96
3	9.90	0.99	30	100	1.692	0.948	9.58
4	10.00	1.00	45	100	1.661	0.931	9.31
5	12.30	1.23	60	100	2.126	1.191	9.68
6	9.95	0.99	90	100	1.499	0.840	8.44
7	9.40	0.94	120	100	1.412	0.791	8.41

TABLE VII

Purine Nitrogen of Yeast Ribonucleic Acid of Merck purified according to Makino's Method

Experiment No.	Yeast ribonucleic acid	Hydrolysis			$N/10$ H_2SO_4 employed (Kjeldahl)	Purine nitrogen	Purine nitrogen per 100 mg. of RNA.
		10% Sulfuric acid	Time	Temperature			
	mg.	*ml.*	*minutes*	°*C*	*ml.*	*mg.*	*mg.*
1	9.92	0.99	100	15	1.513	0.848	8.55
2	11.70	1.17	100	30	1.739	0.975	8.33
3	11.20	1.12	100	45	1.688	0.946	8.45
						Average	8.44

Amino Nitrogen—The aminonitrogens of yeast ribonucleic acid, uncleotides, nucleosides, purines and pyrimidines were determined by Van Slyke's nitrous acid gasometric method (*12*). Since the amino nitrogen of yeast ribonucleic acid, nucleotides, nucleosides, purines and pyrimidines is split very slowly taking for more than several hours, (*12, 13*) connection cock of Van Slyke's amino nitrogen apparatus should be strictly air-tight. During deamination the reactionflask was immersed in water (20°) and readings were made several times for 5–8 hours as usual. To test the completion of the reaction, it is observed that there should be less gas than in blank tests, usually less than 0.1 ml. per 20 ml. of all evolved gas, after absorbing nitric oxide.

In cases of purines and their nucleosides the reaction was completed within 3 hours. Adenine and adenosine reacted normally, but guanine and guanosine regularly yielded 1.12 and 1.33 molecules of nitrogen, respectively, instead of 1 molecule. Cytosine (dissolved in 2 per cent sodium hydroxide solution) yielded 1.33 molecule of nitrogen after 5 hours and 1.36 molecules of nitrogen after 9 hours. Cytidylic acid yielded 1.15 molecules. (Table VIII-XIII)

In case of yeast ribonucleic acid which was neutralized by $N/10$ sodium hydroxide using phenolphthalein as an indicator, the reaction was completed in 11–12 hours yielding 3.09 mg. of amino-nitrogen (average) per 100 mg. of the acid (Table XIV).
The value is 20 per cent higher than that calculated on the tetranucleotide formula. But, when the excess of nitrogen liberated from purines, pyrimidines or their nucleosides are taken into account, this result for yeast ribonucleic acid seems to be in close agreement with that required for the presence of three amino-groups. A 100 mg.-portion of the

TABLE VIII

Amino Nitrogen of Adenine

Experiment No.	Weight mg.	Amino nitrogen (Theoretical value) mg.	Time of reaction hours	Amino nitrogen (Experimental value) mg.	Molecule of amino nitrogen yielded
I	11.17	0.83	2/3	0.13	
			3	0.79	0.95
			6	0.79	0.95
II	11.0	0.815	3	0.86	1.05
			5	0.86	1.05
III	11.0	0.815	2¼	0.69	
			3	0.78	0.96
			4	0.78	0.96

TABLE IX

Amino Nitrogen of Guanine

Experiment No.	Weight	Amino nitrogen (Theoretical value)	Time of reaction hours	Amino nitrogen (Experimental value) mg.	Molecule of amino nitrogen yielded*
I	7.98	0.74	3	0.82	1.11
			4	0.83	1.12
			5	0.81	1.10
II	7.98	0.74	3	0.83	1.12
			4	0.83	1.12
			5	0.83	1.12
III	7.77	0.72	2	0.74	
			3	0.77	
			6	0.81	1.12
			8	0.81	1.12

* Theoretical value is 1 molecule.

TABLE X

Amino Nitrogen of Adenosine

Experiment No.	Weight	Amino nitrogen (Theoretical value)	Time of reaction	Amino nitrogen (Experimental value)	Molecule of amino nitrogen yielded*
	mg.	*mg.*	*hours*	*mg.*	
I	15.61	0.76	5/6	0.70	
			3	0.76	1
			5	0.74	0.97
II	11.28	0.55	3	0.55	1
			4½	0.55	1

TABLE XI

Amino Nitrogen of Guanosine

Experiment No.	Weight	Amino nitrogen (Theoretical value)	Time of reaction	Amino nitrogen (Experimental value)	Molecule of amino nitrogen yielded*
	mg.	*mg.*	*hours*	*mg.*	
I	18.09	0.79	3	1.05	1.33
			4	1.05	1.33
			5.5	1.03	1.31
II	7.80	0.34	3	0.45	1.32
			6	0.46	1.35
III	7.80	0.34	3	0.46	1.35
			7	0.45	1.32

TABLE XII

Amino Nitrogen of Cytosine

Weight	Amino nitrogen (Theoretical value)	Time of reaction	Amino nitrogen (Experimental value)	Molecule of amino nitrogen yielded*
mg.	*mg.*	*hours*	*mg.*	
11.40	1.24	2	1.30	
		3	1.52	1.23
		5	1.65	1.33
		7	1.67	1.35
		9	1.69	1.36

TABLE XIII

Amino Nitrogen of Cytidylic Acid

Weight	Amino nitrogen (Theoretical value)	Time of reaction	Amino nitrogen (Experimental value)	Molecule of amino nitrogen yielded*
mg.	*mg.*	*hours*	*mg.*	
25.84	1.12	5/6	0.049	
		4	0.817	
		7½	1.112	
		12	1.282	1.15
		18½	1.286	1.15

Merck sample contains 12.86 mg. of nitrogen and one yeast ribonucleic acid molecule has 15 atoms of nitrogen calculated on tetranucleotide structure; therefore one atom of nitrogen corresponds to 0.857 mg. The explanation in detail becomes as follows:

Guanosine	$0.857\times\frac{133}{100}=1.140$ mg.
(excess of 33 per cent)	
Cytidylic acid	$0.857\times\frac{115}{100}=0.986$ mg.
(excess of 15 per cent)	
Adenosine	0.857 mg.
Total	2.983 mg.

Table XIV

Amino Nitrogen liberlated by Yeast Ribonucleic Acid of Merck

Experiment No.	Weight	Time of reaction	Amino nitrogen (Experimental value)	Amino nitrogen per 100mg. of RNA.
	mg.	*hours*	*mg.*	*mg.*
I	27.11	3	0.55	
		5	0.76	
		7	0.83	3.06
		9	0.85	3.14
		11	0.85	3.14
II	25.57	3	0.32	
		5	0.53	
		7	0.60	
		8	0.68	
		10	0.74	
		12	0.77	3.05
		14	0.78	3.05
			Average	3.09

Hydrolyzate of yeast ribonucleic acid which was obtained by treating with 10 per cent sulfuric acid at 100° for 45 minutes completed the reaction within 4 hours yielding 2.87 mg. of amino-nitrogen per 100 mg. in a Van Slyke apparatus (Table XV).

TABLE XV

Amino Nitrogen liberated by the Hydrolyte of Yeast Ribonucleic Acid of Merck

Experiment No.	Weight	Time of reaction	Amino nitrogen (Experimental value)	Amino nitrogen per 100mg. of RNA.
	mg.	*hours*	*mg.*	*mg.*
I	20.6	2	0.50	
		4	0.62	2.93
		6	0.60	2.91
II	20.7	2	0.47	
		4	0.59	2.85
		6	0.58	2.80
			Average	2.87

Similarly, the result seems to be in close agreement with the value required from the tetranucleotide formula as follows:

Guanine (excess of 12 per cent)		$0.857\times\frac{112}{100}=0.960$ mg.
Cytosine (excess of 36 per cent)		$0.857\times\frac{136}{100}=1.165$ mg.
Adenine		0.857 mg.
	Total	2.982 mg.

Purine amino-nitrogen was determined by Van Slyke's method as follows. Yeast ribonucleic acid was hydrolyzed by 10 per cent sulfuric acid in boiling water for 45 minutes and its purines were precipitated with ammoniacal silver method and recovered by 25 per cent hydrochloric acid in the same conditions as the guanine determination. The supernatant which contained purines was made alkaline with 33 per cent sodium hydroxide to expell ammonia by evacuation from the supernatant and then neutralized with dilute hydrochloric acid. The resulting solution was measured in the graduated flask and certain volume of it was introduced into the reaction chamber of Van Slyke's apparatus. Desamination of the purine fractions completed within 3 hours at 20° and yielded 1.66 mg. of aminonitrogen per 100 mg. of yeast ribonucleic acid (Table XVI, XVII).

TABLE XVI

Purine Amino Nitrogen of Yeast Ribonucleic Acid of Merck

Experiment No.	Weight	Time of reaction	Amino nitrogen (Experimental value)	Amino nitrogen per 100 mg. of RNA.
	mg.	*hours*	*mg.*	*mg.*
I	37.68	2/3	0.54	
		3	0.63	1.68
		5	0.63	1.68
II	37.68	1	0.47	
		3	0.64	1.70
		5	0.64	1.70
III	35.4	1/2	0.45	
		3	0.58	1.64
		5	0.58	1.64
IV	35.4	1/3	0.38	
		3	0.57	1.62
		5	0.57	1.62
			Average	1.66

DISCUSSION

From the data of purine-N and guanine-N obtained above, the molar ratios of purines can be calculated as follows:

Purine N : Guanine N=2.02:1.00
Adenine N : Guanine N=1.02:1.00
Pyrimidine N : Purine N=1.00:1.91

Adenine nitrogen is calculated from purine and guanine nitrogens and pyrimidine nitrogen is obtained by subtracting the value of the purine nitrogen from that of the total nitrogen. Likewise the molar ratios of the other yeast ribonucleic acid prepared by Bauman's method are as follows:

Purine N : Guanine N=2.01:1.00
Adenine N : Guanine N=1.01:1.00
Pyrimidine N : Purine N=1.00:1.88

TABLE XVII

Amino Nitrogen of Guanine and Adenine recovered from Silver Salt

Experiment No.	Sample weight	Amino nitrogen (theoretical value)	Time of reaction	Amino nitrogen (experimental value)	Error
	mg.	*mg.*	*hours*	*mg.*	*per cent*
I	Guanine 12.83	1.19	1	0.94	
			3	1.26	+5.9
			5	1.25	+5.0
II	Guanine 12.83	1.19	1	0.91	
			3	1.29	+8.4
			5	1.29	+8.4
III	Adenine 11.96	0.885	3	0.89	+0.6
			5	0.89	+0.6
IV	Adenine 11.96	0.885	3	0.88	−0.6
			5	0.88	−0.6

By Van Slyke's nitrous acid gasometric method, the hydrolyzate of yeast ribonucleic acid yielded 2.87 mg. of amino-nitrogen and purine fraction yielded 1.66 mg. of amino-nitrogen per 100 mg. of yeast ribonucleic acid. Therefore, difference between 2.87 and 1.66 mg., *i.e.*, 1.21 mg., corresponds to the amino-nitrogen of cytosine. But by the above method cytosine reacts abnormally and yields 136 per cent of the theoretical amounts of amino nitrogen and hence the real value for cytosine is $1.23 \times \frac{100}{136} = 0.904$ mg. per 100 mg. of the yeast ribonucleic acid. This is in proximate agreement with 0.857 mg., the theoretical value for one amino group.

From evidences which we obtained in the above experiments, it is clear that the individual bases (adenine, guanine, cytosine and uracil) of yeast ribonucleic acid of Merck purified according to Makino occur in nearly equal molar ratios.

SUMMARY

1. The writer proposed the method for determination of guanine which consists of precipitation of purine with ammoniacal silver nitrate

and titration with hypoiodite according to Willstätter and Schudel.

2. From the analytical values of nitrogen, phosphorus, amino-nitrogen (Van Slyke's method), purine-nitrogen (copper bisulfite method) and guanine, the writer suggests that the individual bases (adenine, guanine, cytosine and uracil) in yeast nucleic acid (Merck) occur in nearly equal molar ratios.

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THE ISOLATION OF LITHOCHOLIC ACID FROM HOG BILE

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Five bile acids have already been found in hog bile since it was first examined by Windaus and Bohne (*1*), who isolated hyodesoxycholic acid conjugated with glycine. About ten years later, 3-hydroxy-6-keto-*allo*cholanic acid (*2*, *3*), β-hyodesoxycholic acid (*4*) and chenodesoxycholic acid (*5*) were found. Recently, Trickey (*6*) subjected the azoylamide of the non-crystalline fraction of hog bile to chromatographic separation and obtained a C_{27} acid in addition to three different bile acids above described.

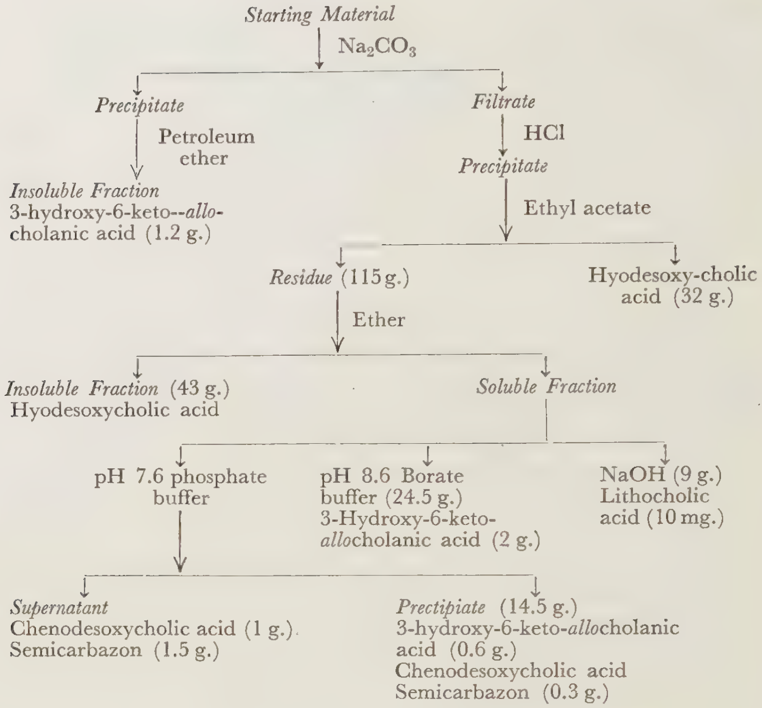
Considering the variety of the hog bile components, it might be possible to find some another bile salts. The present paper reports the further investigation on this point.

Whole hog bile was subjected to alkaline hydrolysis to release unconjugated acids which were recovered as a dark brown resinous mixture. From this bile acids mixture hyodesoxycholic acid precipitated out on addition of ethylacetate. The amorphous fraction after the filtration of hyodesoxycholic acid was dissolved in ether and the ether was exhaustively extracted with the phosphate buffer (pH 7.6), the borate buffer (pH 8.6) and dilute caustic soda solution in succession. The caustic soda fraction, after treatment with petroleum ether, yielded a small amount of lithocholic acid which is already known as the component of hog bile stone (*7*, *8*).

It seems to be interesting from the biogenetic viewpoint that lithocholic acid is contained in hog bile together with hyodesoxycholic acid, β-hyodesoxycholic acid and 3-hydroxy-6-keto-cholanic acid.

EXPERIMENTAL

The whole hog bile was hydrolyzed and the free bile acids mixture was crystallized from ethyl acetate by the usual method. The remaining bile acids mixture after the separation of hyodesoxycholic acid was served


Starting Material
Na2CO3
Precipitate
Petroleum ether
Insoluble Fraction
3-hydroxy-6-keto--allo-cholanic acid (1.2 g.)
Filtrate
HCl
Precipitate
Ethyl acetate
Residue (115 g.)
Hyodesoxy-cholic acid (32 g.)
Ether
Insoluble Fraction (43 g.)
Hyodesoxycholic acid
Soluble Fraction
pH 7.6 phosphate buffer
pH 8.6 Borate buffer (24.5 g.)
3-Hydroxy-6-keto-allocholanic acid (2 g.)
NaOH (9 g.)
Lithocholic acid (10 mg.)
Supernatant
Chenodesoxycholic acid (1 g.)
Semicarbazon (1.5 g.)
Prectipiate (14.5 g.)
3-hydroxy-6-keto-allocholanic acid (0.6 g.)
Chenodesoxycholic acid
Semicarbazon (0.3 g.)

as the material of this experiment.

This material was dissolved in 15 per cent cautic soda solution, diluted with two liters of methanol and heated at 120° in autoclave for 6 hours. After standing overnight the hydrolysate was acidified with dilute hydrochloric acid, leading to the precipitation of bile acids mixture. The mixture was dissolved in 5 per cent soda solution by warming. After standing overnight, there was a voluminous white precipitate which was separated by centrifugation, suspended in dilute hydrochloric acid and extracted with ether. The ether was washed twice with water, dried with sodium sulfate and evaporated to dryness. This residue was separated by repeated treatment with small volumes of petroleum ether into soluble and insoluble fractions.

The soluble fraction yielded after crystallisation from ethanol plate-shaped crystals of m.p. 68°, which gave negative color reaction of bile acid and seemed to be a fatty acid mixture. The insoluble fraction yielded after several recrystallisations from acetone 1.2 g. of 3-hydroxy-

6-keto-*allo*cholanic acid.

The supernantant soda solution after centrifugation was acidified with dilute hydrochloric acid. A gummy mixture of bile acids was separated, filtered, washed and finely divided. After drying at room temperature for several days, 185 g. of brown powder were obtained. This material was treated with ethyl acetate to obtain a crystalline material which gave after recrystallisation from the same solvent 32 g. of hyodesoxycholic acid.

The non-crystalline residue after separation of hyodesoxycholic acid weighed 115 g. This material was dissolved in dilute ammonia. The ammonia solution was put into a separatory funnel, mixed with ether, acidified carefully with dilute hydrochloric acid and shaken vigorously. The ether insoluble precipitate was again dissolved in ammonia, acidified and extracted with ether. This treatment was repeated once again. Finally 43 g. of ether insoluble material remained, which could not be crystallized except that a small amount of hyodesoxycholic acid was obtained after treatment with ethyl acetate.

The ether extracts were united together and treated repeatedly with phosphate buffers at pH 7.6 until a portion of the buffer showed no cloudiness on acidification. Similar treatments with borate buffers of pH 8.6, and then with dilute caustic soda solution followed thereafter.

Phosphate Buffer at pH 7.6—All washings of the phosphate buffer were brought together and heated on a steam bath to remove ether. After standing at room temperature overnight there was a precipitate which was separated by centrifugation.

The supernatant solution was acidified with dilute hydrochloric acid to obtain a bile acids mixture. This mixture, after treatment with semicarbazide to remove keto acid, was dissolved in ether and the ether was repeatedly extracted with small volumes of 0.25 per cent soda solution. Each soda solution was acidified separately, giving an acids mixture. This mixture was formylated and crystallized from dilute alcohol. From middle fractions tiny needles in cluster were obtained, which melted at 184° and showed no depression of the melting point when mixed with the authentic sample of diformyl-chenodesoxycholic acid. It gave a cherry red color with Lieberman's test.

Total Yield: 1 g.

Analysis: $C_{26}H_{40}O_6$, Calculated, C 69.59, H 8.99;
Found, C 69.15, H 9.12.

The precipitate in phosphate buffer of pH 7.6 yielded 14.5 g. of acids mixture. This material was dissolved in absolute ethanol, mixed with a drop of 1 per cent phenolphthalein solution and neutralized with sodium ethylate. Immediately, there appeared needleshaped crystals which were, after treatment with semicarbazide in the usual manner to remove keto acid, formylated and crystallized from dilute alcohol to give a small amount of diformylchenodesoxycholic acid.

The alcoholic mother liquor after filtration of sodium salt crystals was diluted with water, evaporated to remove alcohol. To this saturated common salt solution was added drop by drop, upon which a white finely divided precipitate came out. The addition was stopped as soon as a gummy and brown precipitate began to deposit.

The white bile salts mixture, thus precipitated, was converted into a free acids mixture, which was again dissolved in a small volume of absolute ethanol and treated with sodium ethylate as above described. Immediately were obtained bile salt crystals, which were later worked out in the usual manner to yield 3-hydroxy-6-keto-*allo*cholanic acid.

Borate Buffers of pH 8.6—A portion of 24.5 g. of crude acids mixture were obtained from the extracts with buffer solution at pH 8.6. This material was treated with sodium ethylate in absolute ethanol as above described to yield 2 g. of 3-hydroxy-6-keto-*allo*cholanic acid.

Caustic Soda Solution—An acids mixture obtained from caustic soda extracts was treated with petroleum ether leaving insoluble material behind. This petroleum ether insoluble material was dissolved in a small volume of absolute ethanol, mixed with a drop of 1 per cent phenolphthalein solution and neutralized with sodium ethylate. Long needle-shaped crystals were obtained which were dissolved in water by warming and acidified with acetic acid. There was a white flocculent precipitate, which was filtered, washed with water and crystallized from acetic acid. Several recrystallisations from the same solvent yielded 10 mg. of plate-shaped crystals, which melted at 184° and showed a positive Pettenkofer's and negative Hammarsten-Yamasaki's reactions. It showed no depression of the metlting point in admixture with the authentic sample of lithocholic acid.

Analysis: Sample dried at 110° in vacuo.
$C_{24}H_{40}O_3$, Calculated, C 76.60, H 10.64;
Found, C 76.24, H 10.51.

Methyl ester of this acid was prepared with diazomethane and crystallized from dilute methanol. Long needle-shaped crystals were obtained which melted at 126° and showed no depression of the melting point when mixed with the authentic methyl lithocholate.

SUMMARY

The bile salts of hog were examined and a small amount of lithocholic acid was obtained together with hyodesoxycholic acid, 3-hydroxy-6-keto-*allo*cholanic acid and chenodesoxycholic acid.

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PREPARATION OF 3(β)-HYDROXY-Δ^5-ALLOCHOLENIC ACID FROM HYODESOXYCHOLIC ACID

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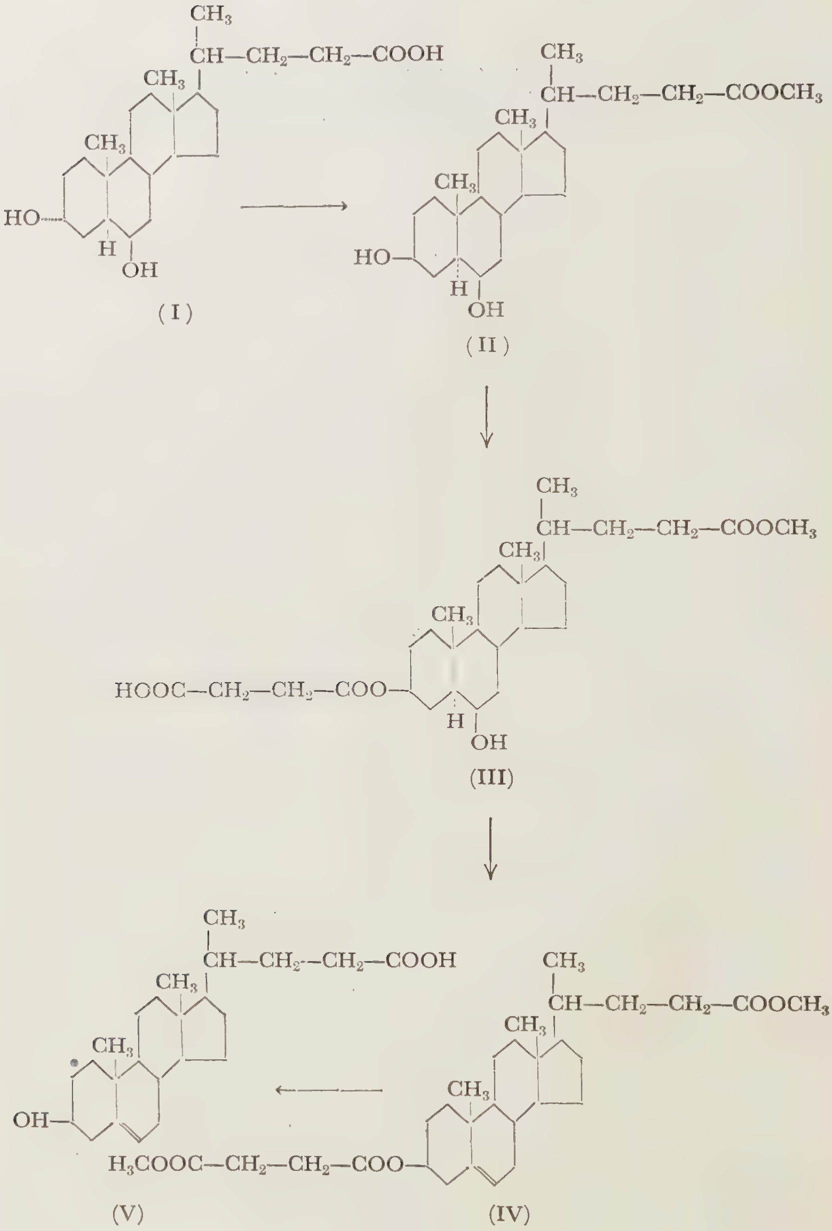
The removal of water molecule from adjacent carbon atoms, leading to the formation of unsaturated compounds, has been familiar in the chemistry of bile acid. One of the most effective procedures to achieve such dehydration consists in distillation in a high vacuum, thus cholic acid being dehydrated to cholatrienic acid (*1*). But this method is so effective that all hydroxyl groups of the starting substance are dehydrated, so that it is quite unsuitable to obtain a partial dehydration product. Sometimes more milder dehydration agents, such as zinc chloride, conc. hydrochloric acid, acetylchloride in acetic acid or potassium bisulfate are used successfully, for instance in cases of dehydroxycholenic acids (*2*) and hydroxycholadienic acid (*3*).

Applying those dehydration methods for hyodesoxycholic acid we can not obtain 3-hydroxy-Δ^5-*allo*cholenic acid. The C_3-hydroxyl group should be protected before treatment with a dehydration agent. For this purpose acetylation was tried, but failed as the C_3- and C_6-hydroxyl groups behaved quite similarly giving only non-acylated or diacylated compound. After many trials the following procedure was found to be successful: Hydexoxycholic acid (I) was first convereted 3(β), 6(β)-dihydroxy-*allo*cholanic acid according to the description of Windaus (*4*).

Then it was methylated to methyl 3(β), 6(β)-dihydroxy-*allo*cholanate (II). Treatment of this substance with succinic anhydride in pyridine gave the mono-succinylated compound (III), the succinyl group being attached to the C_3-hydroxyl. This substance was methylated with diazomethane, dissolved in pyridine and dehydrated with $POCl_3$ to give methyl 3(β)-methoxysuccinylhydroxy-Δ^5-*allo*cholanate (IV). IV was hydrolyzed with alkali to 3(β)-hydroxy-Δ^5-*allocholenic* acid (V), which E.S. Wallis (*5*) had obtained for the first time by the oxidation of cholesterol with chromium trioxide.


CH₃
CH—CH₂—CH₂—COOH
CH₃
CH₃
HO
H
OH
(I)
CH₃
CH—CH₂—CH₂—COOCH₃
CH₃
CH₃
HO—
H
OH
(II)
CH₃
CH—CH₂—CH₂—COOCH₃
CH₃
CH₃
HOOC—CH₂—CH₂—COO—
H
OH
(III)
CH₃
CH—CH₂—CH₂—COOH
CH₃
CH₃
OH—
(V)
CH₃
CH—CH₂—CH₂—COOCH₃
CH₃
CH₃
H₃COOC—CH₂—CH₂—COO—
(IV)

EXPERIMENTAL

Methyl 3(β)-succinylhydroxy-6(β)-hydroxy-allocholanate (III)—200 mg. of methyl 3(β), 6(β)-dihydroxy-*allo*cholanate (prepared from hyodesoxycholic acid according to Windaus (*4*)), 290 mg. of succinic anhydride and 2.7 ml. of dry pyridine were mixed, sealed in a glass tube, heated on a steam bath for two hours and kept at room temperature over night. The tube was opened and the contents were was poured into 100 ml. of ice water containing 2 ml. of conc. sulphuric acid. The solution was extracted with ether. The ether was washed twice with water, dried with sodium sulfate, reduced to a smaller volume by evaporation and kept overnight. 195 mg. of prisms, which melted at 168–172°, were obtained after recrystallisation from ether.

3(β)-*Hydroxy*-6-*keto-allocholanic acid*—A solution of 45 mg. of methyl 3(β)-succinylhydroxy-6(β)-hydroxy-*allo*cholanate (III) in 0.4 ml. of glacial acetic acid was treated with 20 mg. of chromium trioxide in 0.5 ml. of 90 per cent acetic acid. After being kept at room temperature (30°) for 3 hours, 5 ml. of water was gradually added to it. The resulting crystalline oxidation product, was filtered, dissolved in 10 ml. of 5 per cent sodium hydroxide solution and boiled for 2 hours under reflux. After the solution had been cooled, dilute hydrochloric acid was added to make the solution just acid against congo red. The preipitate was filtered, washed with water, dried in a vacuum dessicator and crystallized from dilute alcohol. Recrystallisation from the same solvent gave 25 mg. of needle-shaped crystals, which melted at 237–8° with decomposition and showed no depression of the melting point when mixed with the authentic sample of 3(β)-hydroxy-6-keto-*allo*cholanic acid.

Methyl 3(β)-methoxysuccinylhydroxy-6(β)-hydroxy-*allo*cholanate Methyl 3(β)-succinylhydroxy-6(β)-hydroxy-*allo*cholanate (III) was dissolved in methanol and methylated with diazomethane in the usual manner. The solution which was concentrated to a small volume and kept overnight gave crystals which were recrystallized from the same solvent. Needle-shaped crystals were obtained which melted at 171.5-3°. A mixture with the starting compound (III) melted at 155°.

Analysis: $C_{30}H_{48}O_7$. Calculated, C 69.23, H 9.23,;
Found, C 68.47, H 9.59

Methyl 3(β)-methoxy-succinylhydorxy-Δ^5-allocholenate (IV)—143 mg. of methyl 3(β)-methoxysuccinylhydroxy-6(β)-hydroxy-*allo*cholanate, 1.8ml. of dry pyridine and 0.7 ml. of $POCl_3$ were mixed, sealed in a glass tube, heated on a steam bath for four hours and kept at room temperature for two hours. The tube was opened and the contents was poured into 100 ml. of ice water. After standing overnight, the precipitate was filtered, washed with water, dried in a vacuum dessicator and recrystallized twice from methanol. 75 mg. of elongated recutangular plates were obtained which melted at 141–2°, absorbed bromine promptly and decolorized permanganate solution.

3(β)-Hydroxy-Δ^5-allocholenic acid (V)—A solution of 20 mg. of methyl 3(β)-methoxysuccinylhydroxy-Δ^5-*allo*cholenate (IV) in 8 ml. of 95% ethanol was mixed with 2 ml. of 20 per cent aqueous potassium hydroxide solution, heated under reflux for 2 hours and then 30 ml. of water was added to the reaction mixture. After the solution had been concentrated by distillation to about one third of its original volume to remove the bulk of alcohol, it was allowed to stand at room temperature, acidified with dilute hydrochloric acid and extracted with ether. The ether was washed twice with water, dried with sodium sulfate and allowed to evaporate freely at room temperature. Prismshaped crystals were obtained which after recrystallisation from acetone melted at 235° with decomposition.

Methyl 3(β)-hydroxy-Δ^5-allocholenate—3(β)-Hydroxy-Δ^5-*allo*cholenic acid (V) was dissolved in methanol, methylated with diazomethane and recrystallized from acetone giving needles (m.p., 144°). It absorbed bromine promptly.

Analysis: $C_{25}H_{40}O_3$. Calculated, C 77.32, H 10.31;
Found, C 76.86, H 10.25

SUMMARY

3(β), 6(β)-Dihydroxy-*allo*cholanic acid was prepared from hyodesoxycholic acid and succinylated at the C_3-hydroxyl group. 3(β)-Hydroxy-Δ^5-*allo*cholenic acid was obtained from this succinylated compound by dehydrating the C_3-hydroxyl group with $POCl_2$ and hydrolysing the dehydration product with potassium hydroxide.

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PREPARATION OF 3,12-DIHYDROXY-7-KETO-CHOLANIC ACID

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Kaziro (*1*) as well as other workers studied on the partial oxidation of bile acids and found that three hydroxyl groups in molecule of cholic acid show different susceptibilities against oxidizing agents. Thus he had prepared 3-hydroxy-7,12-diketocholanic acid in good yield by chromic acid oxidation of cholic acid, while 7-keto derivative of cholic acid had been remained unknown until Haslewood (*2*) described the chromate oxidation procedure of cholic acid in acetic acid buffered with sodium acetate. Many partial oxidation procedures (*3*) of cholic acid leading to 7-keto derivative have been reported thereafter. The auther obtained 3,12-dihydroxy-7-ketocholanic acid in good yield by oxidizing cholic acid with permanganate in phosphate buffer solution. In this report its procedure is described.

EXPERIMENTAL

Preparation of 3,12-*Dihydroxy*-7-*ketocholanic Acid*—A 0.2 g. portion of cholic acid was dissolved by warming in a small volume of water containing an equivalent amount of sodium bicarbonate, mixed with 80 ml. of $M/5$ phosphate buffer at pH 7.0 and then 20 ml. of $N/10$ potassium permanganate solution (1.2 moles) were added to the reaction mixture.

The solution was put aside at room temperature for one week. Its red color fainted away gradually and a dark brown precipitate of manganese dioxide appeared, which was filtered off and washed twice with small volume of water. The filtrate and washings were brought together being lastly acidified with dilute hydrochloric acid, upon which needleshaped crystals came out. The Yield was 140 mg. with a mp. of 169°. A recrystallisation from dilute methanol brought the m.p. to 170°. It showed deep red color in contrast to blue one of cholic acid in the Mylius's reaction and gave a strong green fluorescence in the

Hammarsten-Yamasaki's reaction.

Analysis: $C_{24}H_{38}O_5$, Calculated, C 70.88 H 9.42;
Found, C 71.09, H 9.38.

In the following table the yields of 3,12-dihydroxy-7-ketocholanic acid obtained by similar oxidation procedures of 200 mg. of cholic acid at different pH of the buffer solutions are given.

Sorts of Buffer	pH	Yield of 7-keto acid
		mg.
M/5 Phosphate	6.0	64
,,	7.0	140
,,	7.5	122
M/5 Borate	8.0	134
,,	8.5	132

When the oxidation was carried out in borate buffer at pH 9.0, a small amount of cholic acid remained unoxidised.

Preparation of Ethyl 3,12-*Dihydroxy*-7-*keto-cholanate*—3,12-Dihydroxy-7-keto-cholanic acid was ethylated by heating in absolute alcohol containing 1 per cent of sulfuric acid. After usual treatment and recrystallisation from methanol, a crop of small crystals was obtained which melted at 158–9° and showed no depression of the melting point in admixture with a sample prepared from cholic acid according to Haslewood.

Preparation of Desoxycholic Acid—3,12-Dihydroxy-7-keto-cholanic acid in 10 ml. of alcohol were mixed with a solution containing 40 mg. of semicarbazide HCl and 60 mg. of sodium acetate in a small volume of water. The mixture was sealed in a glass tube and heated on a steam-bath for 4 hours. The tube was opened and the contents were poured into a large amount of water. A white precipitate appeared, which after several hours was filtered, washed with water and dried. 60 mg. of amorphous powder were obtained, which could not be crystallized. 50 mg. of this semicarbazone, 0.3 g. of sodium in 2 ml. of absolute alcohol and 2 drops of hydrazine hydrate were brought together, sealed in a glass tube and heated for 6 hours at 180–190°. The tube was opened and the contents were poured into a bulk of water, acidified with dilute hydrochloric acid and kept at room temperature for several hours. The precipitate was filtered, washed with water, dried and crystallized from acetic acid. Elongated prisms weighing 30 mg. was obtained, which melted at 145° and showed no depression of the melting point in admixture with the authentic acetic choleinic acid.

SUMMARY

Cholic acid was oxidized with potassium permanganate in a buffer solution and 3,12-dihydroxy-7-ketocholanic acid was obtained in good yield.

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